VOLUME 35 MARCH 1957 NUMBER 3

Canadian Journal of Biochemistry and Physiology

Editor: K. A. C. ELLIOTT

Associate Editors:

B. D. BURNS, *McGill University*
L.-P. DUGAL, *University of Ottawa*
G. KROTKOV, *Queen's University*
A. G. McCALLA, *University of Alberta*
J. A. McCARTER, *Dalhousie University*
M. NICKERSON, *University of Manitoba*
H. E. RAWLINSON, *University of Alberta*
R. J. ROSSITER, *University of Western Ontario*
A. E. WILHELMI, *Emory University*

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY

(Formerly Canadian Journal of Medical Sciences)

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY and six other journals devoted to the publication, in English or French, of the results of original scientific research. *La Revue accepte des travaux originaux en biochimie, physiologie, pharmacologie, et sujets connexes.*

Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of: members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies. The Canadian Physiological Society has chosen the Canadian Journal of Biochemistry and Physiology as its official journal for the publication of scientific papers.

EDITORIAL BOARD

Representatives of the National Research Council

A. N. CAMPBELL, *University of Manitoba*
G. E. HALL, *University of Western Ontario*
H. G. THODE, *McMaster University*
D. L. THOMSON, *McGill University*
W. H. WATSON (Chairman), *University of Toronto*

Editors of the Journals

D. L. BAILEY, *University of Toronto*
T. W. M. CAMERON, *Macdonald College*
H. E. DUCKWORTH, *McMaster University*
K. A. C. ELLIOTT, *Montreal Neurological Institute*
G. A. LEDINGHAM, *National Research Council*
LÉO MARION, *National Research Council*
R. G. E. MURRAY, *University of Western Ontario*

Representatives of Societies

D. L. BAILEY, *University of Toronto*
Royal Society of Canada
T. W. M. CAMERON, *Macdonald College*
Royal Society of Canada
H. E. DUCKWORTH, *McMaster University*
Royal Society of Canada
Canadian Association of Physicists
K. A. C. ELLIOTT, *Montreal Neurological Institute*
Canadian Physiological Society
R. G. E. MURRAY, *University of Western Ontario*
Canadian Society of Microbiologists
H. G. THODE, *McMaster University*
Chemical Institute of Canada
T. THORVALDSON, *University of Saskatchewan*
Royal Society of Canada

Ex officio

LÉO MARION (Editor-in-Chief), *National Research Council*
F. T. ROSSER, Director, Division of Administration, *National Research Council*

Manuscripts for publication should be submitted to Dr. Léo Marion, Editor-in-Chief, Canadian Journal of Biochemistry and Physiology, National Research Council, Ottawa 2, Canada.

(For instructions on preparation of copy, see **Notes to Contributors** (inside back cover).)

Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration, National Research Council, Ottawa 2, Canada.

Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and prices are:

Canadian Journal of Biochemistry and Physiology	Monthly	$3.00 a year
Canadian Journal of Botany	Bimonthly	$4.00
Canadian Journal of Chemistry	Monthly	$5.00
Canadian Journal of Microbiology	Bimonthly	$3.00
Canadian Journal of Physics	Monthly	$4.00
Canadian Journal of Technology	Bimonthly	$3.00
Canadian Journal of Zoology	Bimonthly	$3.00

The price of single numbers of all journals is 75 cents.

Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 35 MARCH 1957 NUMBER 3

THE ANTIACCELERATOR EFFECT OF VERATRAMINE ON THE ISOLATED PERFUSED HEART OF THE CAT[1]

MAURICE F. MURNAGHAN

Abstract

When isopropylarterenol was continuously infused in the isolated perfused heart of the cat, the heart rate remained at a steady elevated level for more than 2 hours. Veratramine selectively antagonized the accelerator effect of isopropylarterenol, and the dose required to cause a 50% reduction in acceleration (I_{50}) was determined. The mean I_{50} value in 13 experiments (mean of the 13 individual I_{50} values) is 0.415 micromolar (μM) with fiducial limits of 0.182–0.648. Cardiac irregularities, arising from the sinoauricular node, occurred in seven out of 13 experiments after the administration of veratramine. The results indicate that in this preparation coronary flow is related to amplitude but is independent of heart rate.

Introduction

The chronotropic action of the sympathomimetic amines in the heart–lung preparation (HLP) of the dog, the anesthetized dog and cat, and the spinal cat has been shown by Krayer and co-workers (5, 7, 9) to be specifically antagonized by the secondary amine alkaloids and glycosides of the veratrum species. Only in the HLP was the antiaccelerator effect persistent enough to permit quantitative comparison of potency. The results of similar experiments performed on the isolated perfused cat's heart using isopropylarterenol as the accelerator agent are described in this paper.

Methods

Female cats weighing approximately 1.5–3 kg. were used. The cat was rendered unconscious with coal gas, the throat cut, and the heart removed. The extraneous tissues were rapidly excised, and a small incision made into the wall of the left ventricle to confirm that the aortic valves were closed during the perfusion. Perfusion was started within 5 minutes after removal. Recording began approximately 30 minutes later.

The perfusion apparatus consisted of a glass chamber (500 ml. separatory funnel) connected to the cannula by a vertical glass tube 17 cm. in length. The glass chamber was enclosed in a water bath and the vertical glass tube

[1]Manuscript received September 20, 1956.
Contribution from the Department of Pharmacology, University of Ottawa, Ottawa, Ontario. This study was supported by a grant from the National Research Council of Canada.

kept in a glass water jacket connected to the water bath, the water being circulated by a stream of air and thermostatically maintained at 37° C. The fluid in the glass chamber was maintained at a constant level by a Mariotte-bottle. The fluid flowing from the heart was collected by a funnel containing a plug of cotton and passed through a coronary flowmeter into a second funnel. From here the fluid was pumped by a Brewer pipetting machine up into a third funnel where it was filtered through a fast filter paper before passing directly into the glass chamber where it was vigorously oxygenated. Filtration was necessary to prevent coronary vessel occlusion. The volume of circulating Ringer's solution and the perfusion pressure were 200 ml. and 43 cm. respectively. A lever registered amplitude of ventricular contraction on a smoked kymograph. Movement of the lever actuated a Thorp (10) impulse counter for continuous recording of ventricular rate. A Sanborn electrocardiographic machine, connected to needle electrodes in the right auricle, was used for intermittent recording of auricular rate. Coronary flow was measured either by Andrews' (2) or Anderson's (1) flowmeter. While the temperature varied from experiment to experiment within a range of 34.8° C.–36.8° C., care was taken to minimize changes throughout an individual experiment which did not exceed 0.5° C.

Ringer's solution had the following percentage composition: NaCl 0.9, KCl 0.042, $CaCl_2$ 0.024, $NaHCO_3$ 0.015, and dextrose 0.1. Isopropylarterenol hydrochloride was infused into the second funnel at a constant rate by an automatically driven syringe. Infusion rates were usually 0.025–0.05 ml. per minute. The infusion solution was prepared after the heart perfusion had begun by diluting a 0.01% stock solution (pH 1) with 0.9% sodium chloride to give a concentration of 1–2 μg. per milliliter. The acidity prevented deterioration of the isopropylarterenol but produced no significant change in the pH of the Ringer's solution throughout the course of the experiment when the solution was infused at a rate of 0.05 ml. per minute.

Veratramine was injected into the glass chamber in a volume of 0.1–0.8 ml. The base was brought into solution with 0.2 ml. of $N/10$ hydrochloric acid for every 5 mg. of the alkaloid and made up to a concentration of 1:1000 with distilled water. Fresh solutions were prepared every 2 weeks. All doses of isopropylarterenol and veratramine refer to the base.

Results

When isopropylarterenol was administered continuously at a rate of 0.025–0.05 μg. per minute in the isolated perfused cat's heart, the heart rate increased until a steady state was attained and decreased little over a period of 2½ hours. In five experiments the average time until the acceleration had fallen by 10% was 120 ± 8 (S.E.M.) minutes. The average duration of these experiments was 155 ± 9 minutes and the average fall in acceleration at this time was 15 ± 7%.

When veratramine was given to an isolated perfused heart with an elevated rate due to a continuous infusion of isopropylarterenol, the rate fell abruptly in the first 3 minutes and then more slowly until it attained a steady state

10 to 15 minutes later. In the quantitative estimation of the antiaccelerator potency of veratramine the procedure followed that of Krayer (6). The results of a typical experiment are given in Fig. 1 and the data from 13 experiments are included in Table I. In the eight experiments where the minimal rate to which the heart could be brought by veratramine was less than the initial rate, the former was used in the calculation of maximum acceleration and percentage inhibition. The dose of veratramine required to cause a 50% reduction in acceleration, i.e. the I_{50} value, was calculated for each experiment by a probit analysis. The arithmetic mean of the individual I_{50} values is 34.0 μg., with fiducial limits of 15.0–53.1 ($P = 0.05$). As the volume of circulating fluid was 200 ml. and the molecular weight of veratramine 409.6, these figures correspond to micromolar values of 0.415, mean and 0.182–0.648, limits. The mean I_{50} value and its fiducial limits were calculated for the pooled data in a similar manner. The mean is 24.4 μg. or 0.298 micromolar (μM); the respective fiducial limits are 14.4–41.2 and 0.176–0.503. The dose response curve for veratramine in Fig. 2 corresponds to the best fitting straight line obtained in the probit analysis of the pooled data. All statistical treatment followed that of Finney (3).

Cardiac irregularities, consisting of alternating and periodic sinus rhythms, occurred during the assay but did not interfere in the quantitative estimation of veratramine. The incidence and the dose of veratramine required to produce them are shown in Table I. Similar rhythms after veratramine have been previously described by other workers (4).

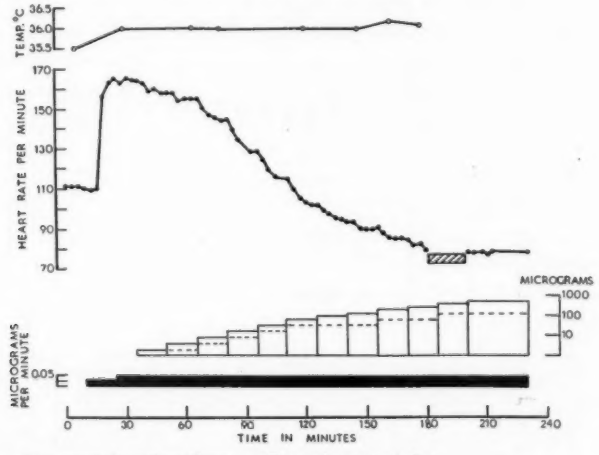


FIG. 1. The graded antiaccelerator effect of veratramine.
Cat, No. 19. Isolated perfused heart preparation. The temperature record is that of the Ringer's solution in the aortic cannula. The hatched area indicates the presence of periodic sinus rhythm. Staircase: dose of veratramine; calibration on right in μg. (log scale). The steps formed by the interrupted lines indicate the dose given; those formed by the uninterrupted lines indicate the total amount present. Black bar: continuous infusion of isopropylarterenol base (1: 1,000,000); calibration on left in μg. per minute.

TABLE I

THE ANTIACCELERATOR EFFECT OF VERATRAMINE IN THE ISOLATED PERFUSED HEART OF THE CAT DURING A CONTINUOUS INFUSION OF ISOPROPYLARTERENOL

Experiment No.	Cat weight, kg.	Heart rate		Heart rate after veratramine		Dose of veratramine, μg.		
		Initial beats/min.	Maximal beats/min.*	Minimal beats/min.	Total decrease beats	Causing maximal effect	Causing irregularities†	I_{50}‡
14	2.16	100	165	75	90	117		17.4
15	1.82	74	132	102	30	46		23.3
16	2.16	75	139	41	98	128		11.5
17	2.30	72	122	60	62	256	A 64 – P 128	12.5
18	2.42	79	140	96	44	32	A 16 – P 32	18.1
19	2.16	108	164	78	86	384	P 126	22.9
20	2.65	133	154	80	74	256	A 384	40.2
21	2.27	105	172	105	67	768		52.2
22	2.05	135	189	134	55	128	A 192	22.6
23	1.82	135	198	111	87	1280		109.4
24	1.60	101	150	80	70	64	P 64	15.4
25	1.60	74	134	65	69	128	P 128	14.3
26	2.84	123	165	124	41	450		82.7

* Isopropylarterenol infusion 0.025 μg./min. in Expts. 14, 18, and 20; 0.05 in remainder.
† A = alternating rhythm; P = periodic rhythm.
‡ Calculated by probit analysis of the individual experiments.

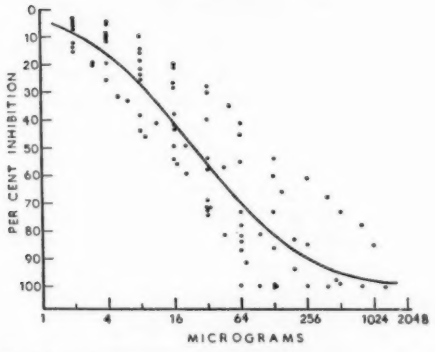


FIG. 2. The antiaccelerator potency of veratramine.
Isolated perfused heart preparation of 13 cats. Cardioacceleration was produced by continuous infusion of 0.025–0.05 μg. of isopropylarterenol base per minute. Ordinate: percentage inhibition of cardioacceleration. Abscissa: doses of veratramine in μg.

In order to investigate the effect of veratramine on amplitude and coronary flow the changes in heart rate, amplitude, and coronary flow were calculated as the mean (and standard error of the mean (S.E.M.)) percentage change from the maximum values recorded during the isopropylarterenol infusion approximately 30 minutes after it began. The values were calculated at two levels; (*a*) at 50% inhibition of acceleration of heart rate and (*b*) at the minimal heart rate. In the control experiments the values were calculated at similar time intervals (95 and 150 minutes), which corresponded to the halfway stage and the end of the experiment respectively. A comparison of the values in the 13 experiments where veratramine was given with the five control experiments shows that while the changes in heart rate are significantly different ($P = 0.001$), the differences in amplitude and coronary flow are not significant. While each of these groups were homogenous regarding heart rate, it was clearly apparent from the data of amplitude and coronary flow that each group consisted of two subgroups; the amplitude and coronary flow decreased in one subgroup (M) but remained constant in the second (Z) in each case (see Table II). The Chi-square test shows that the proportions allocated to subgroups M and Z do not differ significantly among the control and treated groups. Between the two veratramine subgroups these differences in amplitude and coronary flow are significant ($P = 0.01$–0.001), but neither the percentage decrease in rate nor the decrease in beats are significant. While the two control subgroups show similar effects, the small number of experiments do not permit precise comparison within the group. No significant difference exists either between the dose of veratramine in its subgroups or between the times of measurement between groups or subgroups. Table II shows further that the number of experiments in which the amplitude and coronary flow diminish increases with time both in the control and veratramine subgroups.

TABLE II

MEAN (AND S.E.M.) PERCENTAGE CHANGE IN HEART RATE, AMPLITUDE, AND CORONARY FLOW DURING AN ISOPROPYLARTERENOL INFUSION WITH AND WITHOUT VERATRAMINE

	Halfway stage				End of experiment			
Subgroups	No. of expts.	Rate	Amp.	C.F.	No. of expts.	Rate	Amp.	C.F.
Veratramine, M	5	−26 (2.5)	−30 (7)	−48 (9)	7	−47 (5)	−35 (9)	−49 (9)
Control, M	2	− 4 (3.5)	−20 (8)	−28 (4)	4	− 6 (3)	−38 (9)	−32 (10)
Veratramine, Z	8	−21 (1.5)	0 (3)	+ 1 (3)	6	−41 (4)	+ 1 (4)	− 2.5 (2.5)
Control, Z	3	− 1 (1)	+ 3 (1)	0 (4)	1	− 7	+ 6	+ 6

The arrows connect those values between which the '*t*' test shows a significant difference ($P < 0.05$).

Discussion

The observations reported in this paper indicate that the isolated perfused heart of the cat, accelerated by a continuous infusion of isopropylarterenol, may be used as a preparation for study and assay of the antiaccelerator action of veratramine. A comparison of the antiaccelerator potency of veratramine in the perfused cat's heart, using isopropylarterenol as the accelerator agent, with that obtained by Krayer (8) in the HLP of the dog using epinephrine, shows that no significant difference exists between the means from the two methods. Although the product of the S.E.M. by the '*t*' 5% value is 2.5 times greater than in the HLP, the isolated perfused cat's heart is a simpler preparation for estimating antiaccelerator potency.

The results on amplitude and coronary flow show that the proportion of hearts in which these parameters fell increases with time and that this fall is unrelated to heart rate or the concentration of veratramine. These findings suggest that veratramine in this preparation, as in the HLP of the dog, does not inhibit the positive inotropic or coronary dilator effects of the sympathomimetic amines, and that amplitude probably plays a more important role than heart rate in the control of coronary flow or that the coronary dilator and inotropic effects of isopropylarterenol are dependent upon an identical mechanism.

Acknowledgment

The author is indebted to Dr. G. L. Maison, Riker Laboratories Inc., for the generous supply of veratramine and to Winthrop-Stearns Inc. for the sample of DL-*N*-isopropylarterenol hydrochloride.

References

1. Anderson, F. F. and Cameron, A. J. Am. Pharm. Assoc. **39**, 183 (1950).
2. Andrews, W. H. H. J. Physiol. (London), **117**, 45*P* (1952).
3. Finney, D. J. *In* Biological standardization. 2nd ed. *Edited by* J. H. Burn. Oxford University Press, London and New York. 1950.
4. Kosterlitz, H. W., Krayer, O., and Matallana, A. J. Pharmacol. Exptl. Therap. **113**, 460 (1955).
5. Krayer, O. J. Pharmacol. Exptl. Therap. **96**, 422 (1949).
6. Krayer, O. J. Pharmacol. Exptl. Therap. **98**, 427 (1950).
7. Krayer, O. and Ourisson, P. J. Pharmacol. Exptl. Therap. **112**, 341 (1954).
8. Krayer, O., Uhle, F. C., and Ourisson, P. J. Pharmacol. Exptl. Therap. **102**, 261 (1951).
9. Krayer, O. and Van Maanen, E. F. J. Pharmacol. Exptl. Therap. **97**, 301 (1949).
10. Thorp, R. H. Brit. J. Pharmacol. **3**, 271 (1948).

THE EFFECT OF INGESTED CHLORTETRACYCLINE ON SOME HYDROLASES AND ORGANS ASSOCIATED WITH THE DIGESTIVE PROCESS IN GROWING PIGS

I. ASSAY METHODS FOR PROTEASE, AMYLASE, AND CELLULASE ACTIVITY[1]

M. A. A. Vonk, L. W. McElroy, and R. T. Berg

Abstract

Methods are described for the determination of protease, amylase, and cellulase activity of unknown solutions permitting accurate measurements over a wide range of enzyme concentration in a single determination. Regression equations were obtained relating: (*a*) protease concentrations to the optical densities of casein hydrolyzates precipitated with an acetic acid solution (correlation coefficient, $r = -0.9806$; D.F. 70), (*b*) optical densities of starch–iodine complexes formed in starch hydrolyzates to amylase concentrations (multiple correlation coefficient, $R = 0.9918$; D.F. 62), and (*c*) cellulase concentrations to relative viscosities of carboxymethyl cellulose hydrolyzates (correlation coefficient, $r = -0.9554$; D.F. 34). The influence of chlortetracycline on the activity of these hydrolases *in vitro* was investigated and no effect was detected at a level of 5 p.p.m.

Introduction

The effect of antibiotic supplements on rate of growth and efficiency of feed utilization suggests that they may cause improved digestion and absorption of nutrients. Experiments of Burnside and co-workers (3) indicate an ability to digest more feed without a decrease in digestion coefficients resulting from supplementation of swine rations with chlortetracycline, while increased absorption of digested material by chicks receiving chlortetracycline or penicillin supplement was suggested by Sieburth and co-workers (11).

No evidence is available to the authors' knowledge regarding possible effects of ingested antibiotics on the activity of hydrolases associated with the digestive process in growing swine. In order to study the possibility that such effects exist, it was essential to devise rapid and accurate methods for the determination of the activity of typical hydrolases involved in the first stages of digestion. These methods are described in the first part of this paper. In the second part evidence is presented to show that, at low concentration, chlortetracycline in the assay tubes did not affect the accuracy of results obtained.

A. Determination of Protease, Amylase, and Cellulase Activity

Digestion

Digestion was done in Evelyn colorimeter tubes or in 20 × 150 mm. test tubes with a mixture of Pancreatin 3 × USP* and Intestinal Hog Mucosa† as a source of protease; Pancreatin 3 × USP as a source of amylase; and

[1]Manuscript received October 2, 1956.
Contribution from the Department of Animal Science, University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council of Canada.

*National Biochemical Corporation, Cleveland, Ohio.

†Wilson Laboratories, Chicago, Ill.

Enzyme 19* as a source of cellulase. Each tube contained: 8 ml. of substrate solution, 1 ml. of enzyme solution, and 1 ml. of distilled water. The digestion mixture was adjusted to pH 7.0.

Protease Assay

The method of assay for protease activity was adapted from that of Riesen and co-workers (10) in which protease activity is determined by measuring the concentration of undigested casein in partially hydrolyzed digests. A 3% stock solution of casein was prepared by a modification of the method given by Hawk, Oser, and Summerson (6). Thirty grams of casein was stirred with 70 ml. of 0.45 *N* sodium hydroxide and 800 ml. of water. When the casein was in solution the volume was made to 1000 ml. with distilled-water. This stock solution was unstable during storage at 5° C. and as a consequence new solutions were prepared when the reading obtained on a diluted blank after the addition of acetic acid indicated the necessity. Before use, the stock solution was diluted to 0.1% casein with 0.2 *M* phosphate buffer at pH 7.0. The final pH was adjusted to 7.0 with a few drops of 8 *N* sodium hydroxide.

A mixture of pancreatin and intestinal hog mucosa was prepared immediately prior to use by the method of Clandinin (4) in which three parts of pancreatin 3 × USP and one part of intestinal hog mucosa are suspended in distilled water, filtered, and brought to concentrations equivalent to 0.02 to 0.2 mg. of dry enzyme mixture per milliliter.

The digestion mixture was shaken in calibrated colorimeter tubes in an incubator room at 37° C. for 2 hours and cooled in a constant temperature water bath at 25° C. for 30 minutes. Five milliliters of a 4% acetic acid solution was added to precipitate the undigested casein, after which the turbidity was measured immediately by means of an Evelyn photoelectric colorimeter using a 620 mμ filter.

Amylase Assay

The photometric density of the blue starch–iodine complex has been employed by several workers, including Bourne and co-workers (2) and Bird and Hopkins (1), in studies of starch degradation. Based on this procedure a method for the measurement of amylase activity was devised. A 2% starch substrate solution was prepared by stirring 10 g. of soluble starch† with 100 ml. of distilled water and pouring the solution into 375 ml. of boiling phosphate buffer at pH 7.0. The solution was boiled for 1 minute and stirred while cooling to room temperature; 1.2 g. of sodium chloride was added and the solution, after dilution to a final volume of 500 ml., was stored at 5° C. for use 18 to 24 hours after preparation. An enzyme solution containing 0.1 mg. of pancreatin per milliliter was prepared immediately before use and 1 ml. portions of appropriate dilutions were added to assay tubes to give final pancreatin concentrations as shown in Table I.

*Rohm and Haas Company, Philadelphia, Pa.
†Reagent grade, Merck and Company, Inc., Rahway, N.J.

The assay tubes were shaken for 2 hours in an incubator room at 37° ± 0.1° C. Following the hydrolysis period, the samples were diluted 1:200 with distilled water and 10 ml. aliquots were transferred to calibrated colorimeter tubes. One milliliter of a 0.002 *N* iodine solution was added and the photometric density of the starch–iodine complex was determined with an Evelyn photoelectric colorimeter using a 660 mμ filter. The iodine solution was prepared by dissolving 12.7 g. of iodine in 25 ml. of 10 *M* potassium iodide solution (5), making to 500 ml., and diluting 1:100 with distilled water. The final solution was stored in the dark at 5° C., for periods not exceeding 4 weeks, in separate glass-stoppered flasks each of which contained an amount sufficient for one series of determinations. The stability of the iodine solution was checked at each assay by conducting a blank determination.

Cellulase Assay

Since cellulose is insoluble in water, it is not a suitable substrate for measuring cellulase action, especially when the activity is of a low order. Several water-soluble derivatives of cellulose have been studied in order to determine their suitability as substrates (8, 9). Results of these studies indicate that the activity of very small quantities of cellulase may be detected by viscosimetric methods when carboxymethyl cellulose (CMC)* is used as substrate (8).

A 2% CMC stock solution was prepared at pH 7.0 in 0.2 *M* phosphate buffer and stored at 5° C. The substrate solution was obtained by diluting the stock solution 1:10 with distilled water. The cellulase solution was prepared by stirring 5 g. of enzyme 19 in 95 ml. of distilled water for 20 minutes and centrifuging the suspension at 1500 r.p.m. for 30 minutes (7). The supernatant liquid was collected, made to 100 ml., and stored under chloroform at 5° C. Under these conditions the cellulase activity remained constant from the 2nd to the 5th week of storage. Further dilution with distilled water was made to concentrations in the digestion tubes equivalent to 0.0001–0.0004 mg. of dry enzyme 19 per milliliter.

The assay tubes were shaken for 18 hours at 37° C. after which they were held in boiling water for 30 minutes and cooled in a constant temperature water bath at 25° C. for 1 hour. The relative viscosity of the hydrolyzates was then determined in a water bath at 25° C. using Ostwald viscosimeters with flow time of 85–103 seconds for 5 ml. of distilled water.

Accuracy of Assays and Method of Calculating Activity of Enzyme Preparations

The accuracy of the methods was tested by conducting assays on different days using a series of tubes in duplicate to which enzyme preparations of known equivalent of dry enzyme had been added. Enzyme equivalents per tube, together with means and standard errors for assay readings, are listed in Table I for protease and amylase and in Table II for cellulase. Regression equations, relating readings of photometric density or relative viscosity to enzyme concentrations, were derived from the raw data and used

*CMC 50, Hercules Powder Company, Hopewell, Va.

in the estimation of the activity of unknown enzyme solutions. Straight-line regression equations were found to be most accurate for protease and cellulase determinations, whereas a quadratic equation considerably improved the accuracy of the method for amylase estimation. A table relating known concentrations of pancreatin to observed photometric densities was computed from the quadratic equation and employed in estimating the activity of amylase preparations of unknown concentration.

The calculated regression equations for the three hydrolases were:

Protease.—$P = 0.01945 - 0.04477\ D$ (correlation coefficient, $r = -0.9806$; D.F. 70) where P is the concentration of pancreatin–intestinal hog mucosa mixture in mg. per ml. and D the photometric density.

Amylase.—$D = 1.17491 - 228.272\ A + 10920\ A^2$ (multiple correlation coefficient, $R = 0.9918$; D.F. 62) where D is the photometric density and A is the concentration of pancreatin in mg. per ml.

TABLE I

MEANS AND STANDARD ERRORS FOR PHOTOMETRIC DENSITIES OF CASEIN AND STARCH HYDROLYZATES AFTER DIGESTION WITH VARYING LEVELS OF ENZYME[1]

Enzyme conc., mg./ml.	Photometric densities	
	Casein hydrolyzate	Starch hydrolyzate
0.000	0.473 ± 0.0020	1.145 ± 0.0045
0.002	0.389 ± 0.0019	0.781 ± 0.0051
0.003	—	0.623 ± 0.0024
0.004	0.335 ± 0.0016	0.468 ± 0.0013
0.005	—	0.300 ± 0.0016
0.006	0.290 ± 0.0013	0.137 ± 0.0030
0.008	0.237 ± 0.0012	0.030 ± 0.0004
0.010	0.181 ± 0.0017	0.006 ± 0.0016
0.012	0.135 ± 0.0009	—
0.016	0.084 ± 0.0011	—
0.020	0.040 ± 0.0038	—

[1]All means are based on four determinations in duplicate.

TABLE II

MEANS AND STANDARD ERRORS FOR RELATIVE VISCOSITIES OF CARBOXYMETHYL CELLULOSE HYDROLYZATES AFTER DIGESTION WITH VARYING LEVELS OF ENZYME 19

No. determinations	Enzyme conc., mg./ml.	Relative viscosity
12	0.0000	2.33 ± 0.055
4	0.0001	2.14 ± 0.010
4	0.0002	2.04 ± 0.017
4	0.0003	1.96 ± 0.050
12	0.0004	1.93 ± 0.019

TABLE III

INFLUENCE OF CHLORTETRACYCLINE ON THE ACTIVITY OF PROTEASE AND AMYLASE

Digestion period (hr.)	Mean enzyme activity[1]			
	Protease[2]		Amylase[3]	
	Control	Treatment[4]	Control	Treatment[4]
½	3.81	3.76	2.03	2.03
1	5.03	5.14	3.12	3.12
1½	6.25	6.25	4.38	4.39
2	9.95	9.95	5.90	5.90
Enzyme added (μg./ml.)	10	10	6	6

[1] All means are based on eight determinations.
[2] In μg. protease mixture per ml.
[3] In μg. pancreatin per ml.
[4] Chlortetracycline 5 p.p.m.

TABLE IV

EFFECT OF CHLORTETRACYCLINE ON CELLULASE ACTIVITY OF ENZYME 19

Digestion period (hr.)	Mean cellulase activity[1,2]	
	Control	Treatment[3]
4½	0.16	0.16
9	0.25	0.23
13½	0.31	0.32
18	0.39	0.38
Enzyme added (μg./ml.)	0.40	0.40

[1] All means are based on eight determinations.
[2] In μg. enzyme 19 per ml.
[3] Chlortetracycline 5 p.p.m.

TABLE V

ANALYSIS OF VARIANCE OF THE EFFECT OF CHLORTETRACYCLINE ON THE ACTIVITY OF HYDROLASES

Source of variation	*F* values		
	Protease	Amylase	Cellulase
Treatment vs. control	0.20	1.52	2.28
Digestion period	6,737**	27,217**	109**
Interaction	0.06	0.20	1.36

**$P < 0.01$.

Cellulase.—$C = 0.002247 - 0.000963\,V$ (correlation coefficient, $r = -0.9554$; D.F. 34) where C is the concentration of enzyme 19 in mg. per ml. and V the relative viscosity of the hydrolyzate. This equation is accurate only for enzyme concentrations between 0.0000 and 0.0004 mg. per ml.

B. Effect of Chlortetracycline on the Hydrolases During Digestion *in vitro*

Methods

The influence of chlortetracycline on the hydrolases was measured by conducting assays in the presence or absence of this antibiotic. From a review of the literature it appeared that the concentration of chlortetracycline in the small intestine is extremely variable. However, using data of Yacowitz and Bird (12) and Sieburth and co-workers (11), it was calculated that growth response with chicks has been obtained at a concentration of approximately 5 p.p.m.; therefore this concentration was employed in *in vitro* digestion.

In setting up the comparison 8 ml. of appropriate substrate, 1 ml. of enzyme solution, and 1 ml. of water were added to the control tubes while 8 ml. of appropriate substrate, 1 ml. of enzyme solution, and 1 ml. of a 50 p.p.m. solution of chlortetracycline* were added to the treatment tubes. Digestion periods and enzyme concentrations employed will be found in Tables III and IV.

An analysis of variance of the raw data summarized in Tables III and IV is presented in Table V. The analysis indicates that under the conditions employed chlortetracycline at a concentration of 5 p.p.m. had no effect on the activity of any of the hydrolases *in vitro*. In preliminary work it was found that at a level of 100 p.p.m. the antibiotic inhibited protease activity.

Acknowledgment

Constructive suggestions and criticisms offered during the course of this study by Dr. D. R. Clandinin, Professor of Poultry Husbandry, Department of Animal Science, University of Alberta, are gratefully acknowledged.

References

1. Bird, R. and Hopkins, R. H. Biochem. J. **56**, 86 (1954).
2. Bourne, E. J., Haworth, Sir N., Macey, A., and Peat, S. J. Chem. Soc. 924 (1948).
3. Burnside, J. E., Grummer, R. H., Phillips, P. H., and Bohstedt, G. J. Animal Sci. **13**, 184 (1954).
4. Clandinin, D. R. Poultry Sci. **28**, 128 (1949).
5. Fales, H. A. and Kenny, F. Inorganic quantitative analysis. D. Appleton-Century Company, Inc., New York. 1939. p. 467.
6. Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical physiological chemistry. 13th ed. The Blakiston Co., New York. 1954. p. 390.
7. Holden, M. Biochem. J. **47**, 426 (1950).
8. Holden, M. and Tracey, M. V. Biochem. J. **47**, 407 (1950).
9. Reese, E. T., Siu, R. G. H., and Levinson, H. S. J. Bacteriol. **59**, 485 (1950).
10. Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W. J. Biol. Chem. **167**, 143 (1947).
11. Sieburth, J. McN., Jezeski, J. J., Hill, E. G., and Carpenter, L. E. Poultry Sci. **33**, 753 (1954).
12. Yacowitz, H. and Bird, O. D. Poultry Sci. **32**, 966 (1953).

*Aureomycin hydrochloride, Research Division, American Cyanamid Ltd., New York, N.Y.

THE EFFECT OF INGESTED CHLORTETRACYCLINE ON SOME HYDROLASES AND ORGANS ASSOCIATED WITH THE DIGESTIVE PROCESS IN GROWING PIGS

II. PANCREAS—DRY WEIGHT, FAT CONTENT, PROTEASE, AND AMYLASE ACTIVITY[1]

M. A. A. Vonk, L. W. McElroy, J. P. Bowland, and R. T. Berg

Abstract

Results of experiments to investigate the effect of dietary chlortetracycline on pancreas dry weight and on the crude fat content and hydrolase activity of the gland are presented. Chlortetracycline fed at a level of 20 g. per ton of feed caused an increase in rate of gain and efficiency of feed utilization by pigs between the ages of 9 and 17 weeks. An effect on the pancreas was indicated by significant increases in: (*a*) dry weight, (*b*) total protease and amylase activity, and (*c*) amylase activity per gram dry matter of the glands from animals receiving the supplemented ration. The difference remained significant after adjustment for body weight by covariance in the case of amylase activity but not for dry weight or protease activity, indicating that the effect of chlortetracycline on amylase activity was more pronounced than that on rate of gain of pigs, pancreas dry weight, or pancreatic protease activity. No significant difference in the crude fat content of the pancreas attributable to the inclusion of chlortetracycline in the ration was observed.

Introduction

Reports have appeared in the literature which indicate that ingested antibiotics may affect specific animal organs. Calesnick and co-workers (5) observed an increased thyroid weight and a lower I^{131} uptake of this gland per gram body weight when penicillin or chlortetracycline was administered to rats at the rate of 1 mg. per kg. of feed. These results were not confirmed by Libby and Meites, who also failed to observe an antithyroid effect when penicillin was supplemented in chick rations (8). Menge and Conner (9), working with chicks, noted an increased thyroid weight per pound of body weight when chlortetracycline or thiouracil was ingested; however, since thiouracil depressed growth while a growth-promoting action was indicated for the antibiotic, the authors suggested that the mechanism of action of chlortetracycline on the thyroid differed from that of thiouracil.

A greater dry weight of the liver per pound body weight was observed by Burgess and co-workers (4) in chicks fed a ration supplemented with penicillin, but Braude and co-workers (2), working with pigs weighing approximately 150 lb., found no significant differences attributable to dietary chlortetracycline for the weights of liver, kidney, and spleen, adjusted for body weight.

An excellent review of possible explanations for the effects of antibiotics on rate of growth and efficiency of feed utilization is included in a recent monograph by Jukes (7). Since the pancreas plays an important role in

[1]Manuscript received October 2, 1956.
Contribution from the Department of Animal Science, University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council of Canada.

the digestive process in supplying hydrolases associated with the digestion of a variety of nutrients, any enhancement in the growth or activity of this gland might be expected to have a favorable influence on rate and efficiency of gain. The present study was undertaken to evaluate the possible effect of dietary chlortetracycline on the weight and digestive enzyme content of the pancreas in growing swine.

Experimental

Between June and December 1955, 14 Yorkshire and six crossbred Lacombe × Yorkshire, 8- to 9-week-old weanling pigs were paired on a littermate, sex, and weight basis. An additional 12 purebred Yorkshire weanling pigs, paired on a sex and weight basis, were on experiment during February 1956. During the experimental period the pigs were housed in an old pig barn where a positive response to antibiotics had been obtained in previous experiments. Lot 1 received the basal ration shown in Table I, while Lot 2 was fed the same ration supplemented with 20 g. of chlortetracycline* per ton of feed. None of the pigs used had received supplementary antibiotic in the creep feed ration nor was any antibiotic added to the dams' lactation ration.

TABLE I

BASAL RATION FOR LOT 1

Ground barley, lb.	84	Iodized salt, lb.	0.5
Soybean oil meal, lb.	10	Vitamin A, I.U./lb.	500
Linseed oil meal, lb.	5	Vitamin D, I.U./lb.	100
Ground limestone, lb.	0.5	Vitamin B_{12}, mg./lb.	0.003

Four feeding and management procedures were used. These involved ad libitum feeding, paired hand-feeding, starvation for 16–24 hours before slaughter, and slaughter 2 hours after feed was last offered. A detailed outline of the feeding and management treatments employed is reserved for Paper III (12) because differences in these treatments were of more significance in relation to results obtained on intestinal and cecal material than to those secured on the pancreas. Live weights were recorded weekly. Feed consumption by groups was recorded weekly for pigs fed ad libitum, and daily for those that were pair-fed.

At 2, 3, 4, 6, or 8 weeks after being placed on test the pigs were killed by pairs by severing the jugular vein. The pancreas and small intestine were removed immediately after slaughter and brought to a room kept at 5° C., where the pancreas was freed from extraneous tissue and cut into small pieces. The anterior 3-meter section of the small intestine was cleaned, washed, everted, and blotted dry. The mucosa was then scraped off with a microscope slide. After removal of samples for dry matter determinations, the remaining pancreatic tissue and intestinal mucosa were stored at 5° C.

*Aureomycin hydrochloride, Research Division, American Cyanamid Ltd., New York, N.Y.

After 16 to 20 hours of storage a large sample, approximately two-thirds of the pancreas, was homogenized in an electric blender with approximately five times its weight of chilled distilled water. The actual homogenization time was approximately 15 minutes, but in order to prevent an excessive rise in temperature during the process the homogenizer was switched off for 2-minute periods at 2-minute intervals. The homogenate was then centrifuged for 45 minutes at 1500 r.p.m. The supernatant liquid was collected and the process was repeated with the sediment. The two portions of supernatant liquid were mixed and diluted to a volume at which 1 ml. of supernatant (hereinafter referred to as homogenate) corresponded to approximately 0.1 g. of pancreas. The exact concentrations were calculated from the recorded weights and volumes. Homogenates of the intestinal mucosa were prepared in a similar manner, except that the amount of water used was calculated to give a final tissue concentration one-half that of the homogenate derived from the pancreas of the same animal. Procedures described above were carried out in a room held at 5° C. The homogenates were stored under toluene at 1° C. until they were assayed approximately 18 hours after preparation.

The results of preliminary assays showed that a mixture of equal volumes of pancreatic and intestinal mucosa homogenates gave: (*a*) maximum readings for protease activity, and (*b*) the same readings for amylase activity as equivalent dilutions of pancreatic homogenate alone. Consequently assays for protease, amylase, and cellulase activity were performed on mixtures of equal volumes of pancreatic and intestinal mucosa homogenates from the first 10 pairs (seven in the case of cellulase) of experimental pigs. Assays were done by the methods described in Paper I (11) and dilutions of the pancreas–intestinal mucosa homogenates were used to give readings that permitted the application of the regression equations presented in Paper I to estimate the activity of the homogenates from the test pigs. Assays for cellulase were discontinued after no activity had been detected in homogenates from the first seven pairs of animals.

In preliminary trials known concentrations of pancreatin 3 × USP and of intestinal hog mucosa (Paper I) were added to one-half of pancreatic material and to one-half of intestinal mucosa scrapings respectively prior to homogenization, while the other half was homogenized without the addition of any enzyme. The concentrations of the added enzyme preparations were calculated to give a pancreatin – intestinal hog mucosa mixture of three parts to one part when the homogenates were prepared and mixed as described above. The results of assays conducted on mixtures of homogenates from pancreatic material and from intestinal mucosa scrapings to which no enzyme had been added and to which enzyme had been added indicated recoveries of 102% of added protease, and 96% of added amylase.

The pancreas glands from the last six pairs of pigs were used to determine whether any difference existed, with respect to fat content of this gland, between pigs fed the basal ration and those fed the basal ration supplemented

with chlortetracycline. After they were trimmed in the usual manner, the glands were dried and ground to permit accurate sampling. Fat determinations were done by ether extraction of 2 g. samples by the A.O.A.C. (1) method. Dry matter was determined by drying the samples to a constant weight in a vacuum oven at 95°–100° C.

Results

Rate of Gain and Efficiency of Feed Utilization

Data for mean daily gain and efficiency of feed utilization are summarized in Table II. Pigs fed chlortetracycline grew faster and required less feed per pound gain than their controls regardless of whether they were fed ad libitum or were limited in their feed intake to the amount eaten by their pair-mates. Results for the pigs fed ad libitum were not analyzed separately, but when the data for rates of gain of all 16 pairs were combined and analyzed the differences between control and antibiotic-fed pair-mates were significant.

TABLE II

EFFECT OF DIETARY CHLORTETRACYCLINE ON MEAN DAILY GAINS AND FEED REQUIRED PER POUND GAIN

Lot	1	2	Between treatment	
Chlortetracycline	−	+	*F* value	No. pairs
Pairs fed by paired-feeding technique				
Daily gain, lb.	0.71	0.81	22.10**	13
Feed/lb. gain, lb.	3.39	3.04	14.22**	13
Pairs group-fed ad libitum				
Daily gain, lb.	0.52	0.85	†	3
Feed/lb. gain, lb.	3.24	2.76	†	3
All pairs combined				
Daily gain, lb.	0.67	0.81	17.69**	16
Feed/lb. gain, lb.	3.27	2.99	†	16

**$P < 0.01$.
†Not calculated.

Weight of Pancreas; Protease and Amylase Activity of Pancreas

Evidence will be presented in Paper III which indicates that differences in amount of feed eaten by control and supplemented pigs fed ad libitum, as well as the interval of time between the last feed and slaughter, are factors that complicate the interpretation of results for hydrolase assays conducted on intestinal and cecal contents. In an attempt to determine whether these factors influenced the effect of chlortetracycline on either pancreas weight or hydrolase activity of pancreas–intestinal mucosa homogenates, *F* values were calculated for interaction of chlortetracycline supplementation with; (*a*) method of feeding, and (*b*) interval between last feed and slaughter. Since these values were not significant, it was concluded that analysis of

variance of results for all 16 pairs of pigs was valid for comparison of control and treatment pairs with respect to pancreas weight and hydrolase activity. All statistical analyses were done on a within pair basis to remove the high between pair variability in weight which existed owing to the fact that pairs were slaughtered at intervals between 2 and 8 weeks after being placed on test.

As pair-mate pigs receiving a ration supplemented with chlortetracycline grew faster, they were heavier than their controls at slaughter. Highly significant correlations existed between body weight and: (*a*) pancreas dry weight, $r = 0.91$, (*b*) total pancreatic protease activity, $r = 0.67$, and (*c*) total pancreatic amylase activity, $r = 0.60$. An analysis of covariance was done on the data for these factors in an attempt to adjust for within pair differences in body weight. The r values derived for correlations between body weight and protease or amylase activity per gram dry weight of pancreas were not significant so no adjustment was attempted on these data. Mean values obtained, together with the results of analysis of variance, are summarized in Table III.

From Table III it may be noted that:

(*a*) the dry weights of the pancreas glands from the pigs fed chlortetracycline were greater than those of their controls, but when adjusted for body weight the differences were not significant;

(*b*) pair-mate differences in the total protease activity of the pancreas were significant at the 5% level before, but not after, adjustment for live weight of the pigs;

(*c*) the corresponding differences for amylase activity were significant at the 1% level both before and after adjustment for body weight;

(*d*) pair-mate differences in amylase activity per gram dry weight of pancreas were significant at the 1% level.

TABLE III

EFFECT OF DIETARY CHLORTETRACYCLINE ON DRY WEIGHT AND HYDROLASE ACTIVITIES[1] OF THE PANCREAS

Lot	1[2]	2[2]	Between treatment	Necessary F value	
Chlortetracycline	−	+	F value	0.05	0.01
Dry weight, g.	11.7	15.2	15.83	5.12	10.56
Adjusted dry weight, g.	12.5	14.4	3.07	5.32	11.26
Total protease, g.	28.9	44.3	7.90	5.32	11.26
Adjusted total protease, g.	29.3	44.0	3.31	5.59	12.25
Total amylase, g.	29.3	47.3	49.65	5.12	10.56
Adjusted total amylase, g.	30.3	46.2	14.38	5.32	11.26
Protease activity/g. dry weight, g.	2.3	2.9	3.74	5.32	11.26
Amylase activity/g. dry weight, g.	2.3	3.2	14.74	5.12	10.56

[1] In terms of equivalent weights of dry enzyme preparation (three parts pancreatin 3 × USP plus one part intestinal hog mucosa in the case of protease, and pancreatin 3 × USP in the case of amylase) in standard solutions (11).

[2] Means of determinations for 10 pigs with the exception of the data for protease activity which are means of determinations for nine pigs.

Fat Content of Pancreas

The mean crude fat content of the pancreas glands of six pairs of pigs placed on test after the above experiments were completed was 14.6% for both the controls and those supplemented with chlortetracycline. Furthermore, it was found that the fat content of the pancreas was not significantly correlated with body weight. These results indicate that it is highly improbable that differences existed in the fat content of the pancreas glands from control and antibiotic-fed animals of the first 10 pairs.

Discussion

Reports in the literature do not agree concerning growth response obtainable from antibiotic supplementation of the ration when feed intake is restricted. Experiments with swine reported by Brown and co-workers (3) and with chicks by Scott and co-workers (10) indicate that the growth response to dietary antibiotics is mainly the result of increased feed intake, whereas Davis and Briggs (6) obtained an increased rate of gain in chicks and poults with antibiotic supplements when feed intake was limited to the amounts consumed by the non-supplemented lots. In the present experiments the average daily gain for three pigs self-fed the basal ration supplemented with chlortetracycline was 63% greater than that of their controls, while the corresponding mean difference in favor of the supplemented ration was 14% for the 13 pairs fed under the limited paired-feeding technique.

The data in Table III show that animals receiving dietary chlortetracycline had larger pancreas glands and greater stores of pancreatic protease and amylase than had their controls. The fact that after adjustment for body weight the differences were not significant for pancreas dry weight or protease activity indicates that the effects of the ingested antibiotic on rate of gain, pancreas dry weight, and pancreatic protease were of similar magnitude.

In the case of amylase, however, the observed differences between test and control pair-mates are not explainable on a basis of increased rate of gain and final size of animal because these differences remained significant after adjustment to a basis of equal body weights. This conclusion is confirmed by data in the last line of Table III which show that the pancreas glands of pigs fed the antibiotic contained significantly more amylase per gram dry matter than did those of their pair-mates. These observations suggest that favorable changes in both the rate of growth of the pancreas and the rate of amylase production per unit mass of this gland were associated with the feeding of chlortetracycline.

References

1. Association of Official Agricultural Chemists. Official methods of analysis. 8th ed. Washington, D. C. 1955. p. 386.
2. Braude, R., Coates, M. E., Davies, M. K., Harrison, G. F., and Mitchell, K. G. Brit. J. Nutrition, **9**, 363 (1955).
3. Brown, P. B., Becker, D. E., Card, L. E., and Terrill, S. W. J. Animal Sci. **10**, 1042 (1951).
4. Burgess, R. C., Gluck, M., and Laughland, D. H. Poultry Sci. **32**, 444 (1953).

5. CALESNICK, B., HARRIS, W. D., and JONES, R. S. Science, **119**, 128 (1954).
6. DAVIS, R. L. and BRIGGS, G. M. Poultry Sci. **30**, 767 (1951).
7. JUKES, T. H. Antibiotics in nutrition. Medical Encyclopedia, Inc., New York. 1955.
8. LIBBY, D. A. and MEITES, J. Science, **120**, 354 (1954).
9. MENGE, H. and CONNER, M. H. Proc. Soc. Exptl. Biol. Med. **88**, 216 (1955).
10. SCOTT, H. M., GOFFI, E. A., and GLISTA, W. A. Poultry Sci. **31**, 751 (1952).
11. VONK, M. A. A., MCELROY, L. W., and BERG, R. T. Can. J. Biochem. Physiol. **35**, 181 (1957).
12. VONK, M. A. A., MCELROY, L. W., and BERG, R. T. Can. J. Biochem. Physiol. **35**, 195 (1957).

THE EFFECT OF INGESTED CHLORTETRACYCLINE ON SOME HYDROLASES AND ORGANS ASSOCIATED WITH THE DIGESTIVE PROCESS IN GROWING PIGS

III. PROTEASE, AMYLASE, AND CELLULASE ACTIVITY OF THE CONTENTS OF THE SMALL INTESTINE AND OF THE CECUM; WEIGHTS OF SMALL INTESTINE AND MUCOSA[1]

M. A. A. Vonk, L. W. McElroy, and R. T. Berg

Abstract

Four treatments, involving differences in method of feeding and interval between last feed and slaughter, were employed in a study of the effect of dietary chlortetracycline on protease, amylase, and cellulase activity in the intestinal and cecal contents of 16 pairs of weanling pigs. Most consistent results were obtained with six pairs which were limited pair-fed except for the final feeding during which feed was available ad libitum for a 4-hour period ending 18 hours before slaughter. The mean total activities of all three hydrolases in the contents of the small intestines and of the ceca of the antibiotic-fed animals of these six pairs were significantly greater than in those of the control animals. Expressed as activity per gram dry matter of intestinal contents, significantly higher values for protease and amylase, but not for cellulase, were observed in the pigs that had received chlortetracycline. When the combined results obtained from all 16 pairs of the experimental animals were analyzed, the results showed that on a basis of activity per gram dry matter of intestinal contents, ingested chlortetracycline was associated with significant increases in amylase and cellulase but not in protease activity. Protease, amylase, and cellulase activities per gram dry matter of cecal contents were higher for pigs fed the antibiotic than for their controls. The mean wet weight of the empty small intestine and the mean dry weight of the mucosa scraped from the anterior 3-meter section of the small intestine were lower for the chlortetracycline-fed animals, but the differences were not statistically significant.

Introduction

Reports are available to indicate that ingested antibiotics may, through their effect on cellulolytic rumen microorganisms, influence cellulose disgestion in ruminants. However, to the authors' knowledge, the possibility that ingested antibiotics may modify the activity of enzymes present in the lumen of the intestine has not been investigated.

In Paper II (7) evidence was presented to show that dietary chlortetracycline increased protease and amylase content of the pancreas. Enzymes from the pancreas, the intestinal mucosa, and microorganisms present in the digestive tract are chiefly responsible for protein and carbohydrate digestion in the small intestine and cecum. The main purpose of the present study was to determine whether differences in protease, amylase, or cellulase activity could be demonstrated between homogenates prepared from intestinal or cecal contents of pigs fed chlortetracycline at a level of 20 g. per ton of ration, and those prepared from control pigs not fed the antibiotic.

From results obtained with chicks, Coates and co-workers (2, 3) have suggested the possibility that, as a result of a lower level of infection in the intestines of animals fed antibiotics, the intestinal wall may be thinner and

[1]Manuscript received October 2, 1956.
Contribution from the Department of Animal Science, University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council of Canada.

thus presumably absorption may be more efficient than in animals not fed antibiotics. In this connection a minor objective of the work reported here was to determine whether differences might be demonstrable between control pigs and those fed chlortetracycline with respect to (*a*) wet weight of the small intestine, and (*b*) dry weight of scrapings from the mucosa of the anterior 3 meters of the small intestine.

Experimental

Materials and Methods

Material for assay was collected from the 16 pairs of pigs used for the study of the effects of dietary chlortetracycline on the pancreas as described in Paper II. The small intestine and cecum were excised and brought to a room kept at 5° C. where the contents were expelled, a sample taken for a dry matter determination, and the remainder stored for 18 to 20 hours before homogenization at 5° C. in electric blenders. Homogenates were prepared by the procedure described for pancreatic homogenates (7) at a concentration of 1 g. of wet intestinal or cecal contents in 5 ml. of distilled water. Prior to assay, homogenates were stored under toluene at 1° C. for 18 hours in the case of protease and amylase, and for 2 hours in the case of cellulase. Protease, amylase, and cellulase assays were conducted on appropriate dilutions by the methods described in Paper I (6).

In connection with the viscosimetric method of cellulase assay employed, it was found that the relative viscosities of mixtures of 1 ml. of dilutions of homogenates of intestinal or cecal contents and 9 ml. of water were the same after as before incubation for 18 hours at 37° C. It was also found that after tubes containing 8 ml. carboxymethyl cellulose and 1 ml. of enzyme 19 extract were incubated for 18 hours, the addition of 1 ml. of fresh homogenate of a given concentration produced the same increase in flow time through Ostwald viscosimeters as did the addition of 1 ml. of the same homogenate to 9 ml. of water. From these observations it was concluded that, in the assay method, correction for the increase in viscosity attributable to the homogenate per se could be made by calculating relative viscosity as follows:

Flow time of 5 ml. aliquot of an 18-hour hydrolyzate of 8 ml. carboxymethyl cellulose solution + 1 ml. homogenate dilution of concentration X + 1 ml. water ÷ flow time of 5 ml. aliquot of freshly prepared mixture of 9 ml. water + 1 ml. homogenate of concentration X.

Recovery Trials

In preliminary trials, known concentrations of reference protease, amylase, and cellulase preparations as described in Paper I were added to one-half of the contents of the small intestines and of the ceca of two pigs prior to homogenization, while the other half was homogenized without the addition of any enzyme. The results of assays conducted on these homogenates indicated recoveries of added hydrolases as follows: (1) from intestinal material —96% for protease and amylase, and 97% for cellulase; (2) from cecal material—101% for protease and amylase, and 96% for cellulase.

Preslaughter Treatment of Experimental Pigs

The selection of a satisfactory time interval after the ingestion of feed at which to kill the experimental animals and collect intestinal and cecal contents for assay presents some problems. Studies of gastrointestinal movements in young swine by Neimeier, as summarized by Dukes (4), indicated that, for about 1 hour after feeding, ingesta were passed relatively rapidly from the stomach to the duodenum to the jejunum. The jejunum was usually empty within 8 to 12 hours of feeding. Ileal motility diminished about 5 hours and cecal motility between 6 and 8 hours after feed was offered. Balls of feces began to leave the colon 14 to 16 hours after feed was given.

It may be concluded from existing knowledge of mechanisms controlling rate of secretion of hydrolases active in the intestine that the rate of secretion of these enzymes would be highest during periods shortly after food is eaten and during which ingesta are moving rapidly through the upper regions of the small intestine. Slaughter of the experimental animals at this time might thus be expected to permit the collection of material from the small intestine with a higher total enzyme activity, but not necessarily a higher activity per unit dry matter than might be observed in intestinal contents from animals slaughtered some hours after they had eaten. It is even more difficult to theorize regarding the selection of an interval between final feeding and slaughter which might be expected to allow for the collection of cecal contents for which assay results would be of most significance in relation to digestion in the live animal. For these reasons, and others discussed below, the intestinal and cecal contents assayed in this study were collected from variable numbers of pairs of pigs which had been subjected to four different treatments.

Treatment A

Three pairs of weanling pigs fed ad libitum were starved for 22–23 hours before being slaughtered by pairs after they had been on test 2, 4, or 6 weeks. Under this treatment the pigs fed chlortetracycline were about 30% heavier at slaughter than their pair-mates, while the dry weights of the contents of their intestines and ceca were approximately twice as great. Since it was obvious that both these factors would complicate interpretation of enzyme activity assays, the practice of self-feeding was abandoned.

Treatment B

The two pairs of weanling pigs in this treatment were hand-fed, the daily feed allowance for pair-mates being limited to the amount consumed the previous day by the mate consuming the least. At 22–23 hours before slaughter after 2 or 4 weeks on test, each animal of a pair was offered a final feed in the amount of one-half that consumed the previous day. It was anticipated that by this procedure it might be possible to reduce the differences between pair-mates observed in *Treatment A* with respect to live weight and weights of intestinal and cecal contents.

Within pair differences in body weight were reduced considerably, but the mean dry weight of intestinal contents of the control pigs was 67% greater than that of their mates while the dry weight of the cecal contents from the pigs fed chlortetracycline was 52% more than that of their controls. It was concluded that these differences were attributable to the fact that although both members of a pair were offered their final feed at the same time, the pigs receiving the antibiotic had eaten all their feed at least 2 hours before the control animals had finished theirs.

Treatment C

Five pairs of 8- to 9-week-old pigs, fed by the limited paired-feeding technique described above, were killed at 2, 4, and 6 (two pairs) or 8 weeks, after being offered one-half the weight of feed consumed the previous day at 22–23 hours, plus approximately one-sixth of this amount at 2 hours, before slaughter. Under this treatment extreme variability was observed within and between pairs in weight of intestinal contents, and in the total as well as in the per gram dry weight, protease, amylase, and cellulase activities of this material. Collection of assay material from pigs slaughtered during a highly active stage of digestion was therefore discontinued.

Treatment D

The six pairs of weanling pigs in this group were limited pair-fed except for the final feeding when they were fed ad libitum for 4 hours, the feed being removed 18 hours before slaughter. In order to minimize differences in body weight between animals slaughtered at different times, three pairs were killed at 3 weeks and three pairs at 4 weeks after they were put on test. Under this treatment the average dry weights of the intestinal contents of control and antibiotic-fed pigs were equal, while, presumably owing to the fact that the pigs receiving chlortetracycline ate more at the final ad libitum feeding, the average weight of their cecal contents was 36% higher than that of the controls. The mean difference in live weight at slaughter was only 1 lb. in favor of the pigs fed the antibiotic. The variability in hydrolase assay results for samples collected from the small intestine was much lower than that for samples obtained under *Treatment C*.

Results

Contents of Small Intestine

In Table I the mean results are shown for the six pairs of pigs in *Treatment D* and for all 16 pairs in *Treatments A*, *B*, *C*, and *D*. In both cases the data were analyzed by the paired *t* test. The results for *Treatment D* indicate that, expressed either as total activity or as activity per gram dry weight, more protease and amylase were present in the intestinal contents of pigs fed chlortetracycline than in those of their controls. Although the total cellulase activity of the intestinal contents of the test animals was significantly greater than that of the controls, this difference was not significant when calculated to a per unit dry matter basis.

TABLE I

EFFECTS OF DIETARY CHLORTETRACYCLINE ON HYDROLASE ACTIVITIES[1] IN CONTENTS OF THE SMALL INTESTINE

Treatment	*D*			*A, B, C, D*		
Lot	1	2	2–1	1	2	2–1
Chlortetracycline	−	+		−	+	
No. pigs	6	6		16	16	
Mean dry weight intestinal contents, g.	19.6	19.4	−0.2	36.5	35.3	−1.2
Mean total protease, g.	7.6	9.7	2.1*	20.3	18.8	−1.5
Mean protease/g. dry weight, mg.	401	504	103 *	456	503	47
Mean total amylase, g.	0.9	1.5	0.6***	2.1	2.3	0.2
Mean amylase/g. dry weight, mg.	51	77	26 **	53	66	13 *
Mean total cellulase, mg.	3.1	4.8	1.7**	7.4	10.2	2.8*
Mean cellulase/g. dry weight, mg.	0.2	0.3	0.1	0.2	0.3	0.1**
Protease/g. dry wt.	8.9	6.5	−2.4	9.9	7.5	−2.4*
Amylase/g. dry wt.						

[1]In terms of equivalent weights of dry enzyme preparation (three parts pancreatin 3 × USP plus one part intestinal hog mucosa in the case of protease, and pancreatin 3 × USP in the case of amylase) in standard solutions (6).

*$P < 0.1$.
**$P < 0.05$.
***$P < 0.01$.

It is of interest to note that despite the great variability of the assay results obtained on intestinal contents collected under different treatments, the results of the analysis on the combined data indicate the existence of higher levels of amylase and cellulase per unit dry matter of intestinal contents from the pigs fed the antibiotic. For all 16 pairs of pigs the mean protease activity per gram dry matter of intestinal contents was approximately 10% higher for the pigs fed the antibiotic than for the controls, but this difference did not prove to be significant.

The observed degree of increase in hydrolase activity per gram dry matter of contents of the small intestine associated with the feeding of chlortetracycline was approximately twice as great for amylase as for protease. A paired *t* test on the combined results for all animals expressed as a ratio, protease activity per gram dry weight: amylase activity per gram dry weight, indicated significance for the probability that the effect of dietary chlortetracycline on amylase activity was more favorable than on protease activity in the small intestine.

Contents of the Cecum

The mean dry weights, hydrolase activities, and results of paired *t* test analyses on data for cecal material are summarized in Table II. Regardless of the preslaughter treatment, the weights and total hydrolase activities

TABLE II

EFFECTS OF DIETARY CHLORTETRACYCLINE ON HYDROLASE ACTIVITIES[1] IN CONTENTS OF THE CECUM

Treatment	*D*			*A, B, C, D*		
Lot	1	2	2–1	1	2	2–1
Chlortetracycline	−	+		−	+	
No. pigs	6	6		16	16	
Mean dry weight cecal contents, g.	26.6	36.2	9.5	22.3	29.6	7.7***
Mean total protease, mg.	368	661	293 **	309	544	235 ***
Mean protease/g. dry weight, mg.	13.9	19.0	5.1	15.1	20.0	4.9**
Mean total amylase, mg.	49	136	87 **	43	121	78 ***
Mean amylase/g. dry weight, mg.	1.9	3.8	1.9**	2.1	4.6	2.5**
Mean total cellulase, mg.	44	105	61 **	37	88	51 ***
Mean cellulase/g. dry weight, mg.	1.9	2.9	1.0**	1.8	2.9	1.1***

[1]As in Table I.
**$P < 0.05$.
***$P < 0.01$.

of cecal contents of animals fed chlortetracycline were greater than those of their controls. Possible explanations for the greater weight of cecal material in pigs fed the antibiotic have been mentioned in the descriptions of the four preslaughter treatments employed.

On a per gram dry matter basis, increases in enzyme activity were significant for protease, amylase, and cellulase when the results for all 16 pairs were combined. When the data for samples collected under *Treatments C* and *D* were analyzed separately, increases in protease and cellulase activity per unit dry matter were found to be significant at the 10% level in the case of *Treatment C*, while for *Treatment D* corresponding increases in amylase and cellulase activity were significant at the 5% point. Protease and amylase activities per gram dry matter of cecal contents were much smaller than those observed for contents of the small intestine, whereas the cellulase activity of cecal contents was much greater than that of intestinal contents.

Weights of Small Intestines and of Mucosa from the Anterior Three Meters

After adhering tissue had been excised from the exterior wall of the intestines and the contents expelled by light pressure between the thumb and forefinger, the intestines were cut into 3- to 4-ft. lengths and flushed carefully with distilled water to remove remaining particles of ingesta. The combined weight of the sections was taken after they had been hung up to drain for 2 hours at 5° C. The anterior 3-meter sections were everted, blotted dry, and scraped with the edge of a microscope slide to remove the mucosa. Dry matter determinations were made by drying samples to a constant weight in a vacuum oven at 95°–100° C. Results obtained are summarized in Table III.

TABLE III

EFFECTS OF DIETARY CHLORTETRACYCLINE ON WET WEIGHT OF SMALL INTESTINE AND ON DRY WEIGHT OF MUCOSA

Lot	1	2	Between treatment
Chlortetracycline	−	+	*F* value[3]
Wet weight small intestine[1], g.	1028	996	0.38
Adjusted wet weight small intestine, g.	1060	964	0.77
Dry weight intestinal mucosa[2], g.	9.9	9.0	0.78
Adjusted dry weight intestinal mucosa, g.	10.4	8.4	0.76

[1]Means for 16 pairs.
[2]Means for 10 pairs from *Treatments A*, *B*, and *C*.
[3]All values non-significant.

Results

Analyses of the data indicated the existence of a highly significant correlation ($r = 0.68$) between body weight and wet weight of small intestine, and of a significant correlation ($r = 0.55$) between body weight and dry weight of mucosa from the anterior 3 meters of the intestine. The results obtained for these characteristics were therefore adjusted for body weight by analysis of covariance. On the adjusted basis the mean wet weight of the intestine was approximately 10%, and the mean dry weight of mucosa approximately 20% less for the pigs fed the antibiotic than for the controls. These differences were not significant at the 10% point.

Discussion

Data indicating increased content of protease and amylase of the pancreas glands of pigs fed chlortetracycline at a level of 20 g. per ton of ration were presented in Paper II. As reported above, dietary chlortetracycline was also associated with higher levels of protease and amylase in the contents of the small intestine and of the cecum. Increased production by the pancreas might account for the higher levels of these enzymes observed in the intestinal and cecal contents of the antibiotic-fed pigs.

The fact that homogenates of intestinal and especially of cecal material from pigs fed chlortetracycline exhibited higher cellulase activity than did those from their controls suggests the interesting possibility that, contrary to what might be expected from the results of most *in vitro* studies regarding the effect of antibiotics on cellulolytic activity of rumen microorganisms, dietary chlortetracycline may stimulate the production of cellulases by microorganisms in the digestive tract of swine. However, other hypotheses may be advanced to explain the observed increases in cellulase activity of cecal contents of the pigs fed chlortetracycline. For example, if more complete digestion and absorption of starch and protein are assumed, increased cellulase production could arise from an adaptation of cellulolytic microorganisms to a resulting higher concentration of cellulose.

A number of workers (1, 5, 8) have reported small increases in carcass fat associated with the feeding of antibiotics to swine. Since the deposition of fat in this species normally depends largely on the digestion, absorption, and metabolism of starchy feed, it is possible that a tendency toward increased carcass fat in antibiotic-fed hogs may be associated with an especially favorable effect of antibiotics on amylase levels in the digestive tract.

It is recognized that data from the limited number of *in vitro* studies presented in Papers II and III probably do not provide an accurate reflection of digestive processes in the intact animal. Nonetheless they do furnish some evidence in favor of the hypothesis that part of the favorable effect of dietary antibiotics, as measured by rate of gain, may be due to a favorable effect on digestive enzyme production.

References

1. BOWLAND, J. P. and MCELROY, L. W. Univ. of Alberta Press Bull. 40, No. 2 (1955).
2. COATES, M. E. Chemistry & Industry, **31**, 1333 (1953).
3. COATES, M. E., DAVIES, M. K., and KON, S. K. Brit. J. Nutrition, **9**, 110 (1955).
4. DUKES, H. H. The physiology of domestic animals. 6th ed. Comstock Publishing Company, Inc., Ithaca, N.Y. 1947. p. 369.
5. PIERCE, E. A. J. Animal Sci. **13**, 338 (1954).
6. VONK, M. A. A., MCELROY, L. W., and BERG, R. T. Can. J. Biochem. Physiol. **35**, 181 (1957).
7. VONK, M. A. A., MCELROY, L. W., BOWLAND, J. P., and BERG, R. T. Can. J. Biochem. Physiol. **35**, 187 (1957).
8. WALLACE, H. D., MILICEVIC, M., PEARSON, A. M., CUNHA, T. J., and KOGER, M. J. Animal Sci. **13**, 177 (1954).

SEASONAL FLUCTUATIONS IN BLOOD VOLUME[1]

J. Doupe, M. H. Ferguson, and J. A. Hildes

Abstract

The plasma volume was measured by T1824 dilution in groups of normal young male subjects at regular intervals over the course of 3 years. The plasma volume increased during the summer and decreased during the winter, but this change was variable from subject to subject and was not as marked during 1 year as during the other 2. Ascorbic acid in daily supplement of 400 mg., taken during the first year of the study, did not influence the plasma volume fluctuations. The red blood cell mass, circulating haemoglobin, and total circulating plasma protein varied in the same way and the mean corpuscular haemoglobin concentration also increased during the summer in 2 of the 3 years. The changes in plasma volume are attributed to the influence of environmental temperature and indicate some degree of seasonal acclimatization. Serial measurements of total body water and extracellular space on a few subjects suggest that the seasonal fluctuation affects mainly the vascular system.

Introduction

The original observations of Barcroft *et al.* (2) that exposure to a high environmental temperature for a day or longer causes an increase in blood volume has been amply confirmed (4, 11, 8, 29). Therefore it is to be expected that the blood volume will show seasonal variations provided that the environmental temperature to which the individuals are exposed varies over a sufficiently wide range. Maxfield *et al.* (19) reported an average increase in four subjects of 22% during the "transition from a cold winter to a mild spring" in Philadelphia, while Sjöstrand (27) referred to observations on four subjects in Stockholm showing the blood volume to increase in the spring and to decrease in the autumn. Brown *et al.* (7) stated that the plasma volume in Eskimos in northern Canada was high throughout the summer but decreased during July and August. This was attributed to a secondary readjustment following a large initial increase. On the other hand, Bass and Henschel (3) commented on the lack of general agreement regarding seasonal variation in body fluids.

The present study, which was carried out over a 3-year period in Winnipeg, was undertaken to ascertain if seasonal changes occurred in plasma and blood volume with any degree of regularity in residents of the north central plain of North America, while living under ordinary protected conditions. Some observations were also made on other blood constituents and on extravascular fluid compartments.

Methods

Subjects

The subjects were all male medical students in good health. No change was made in their usual living habits or clothing. They therefore may be described from mid-September to mid-May as urban, sedentary workers,

[1]Manuscript received November 9, 1956.
Contribution from the D.R.B. Arctic Medical Research Unit, Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Manitoba. This work was aided by a grant from the Defence Research Board of Canada.

TABLE I

THE SUBJECTS AND THEIR MEAN PLASMA VOLUMES

Group	Period	No.	Age, years	Height, cm.	Mean weight, kg.	Mean plasma volume			
						Milliliters	Ml./kg.	Ml./cm.	Ml./m.²
A	1953–54	18	22.4 (20–26)	175 (166–187)	75.1 (55.7–92.5)	3121 ± S.D. 228 (2701–3538)	42.0 ± S.D. 4.58 (35.8–51.1)	17.78 ± S.D. 1.12 (16.27–20.28)	1653 ± S.D. 102 (1482–1937)
B	1953–54	13	24.6 (20–32)	179 (175–189)	75.3 (59.4–92.5)	3073 ± S.D. 308 (2558–3688)	41.4 ± S.D. 5.53 (31.3–50.1)	17.16 ± S.D. 1.56 (14.37–20.03)	1602 ± S.D. 143 (1375–1865)
C	1954–55	14	21.7 (20–29)	176 (161–188)	74.8 (63.0–90.2)	3100 ± S.D. 379 (2665–4041)	41.7 ± S.D. 5.34 (34.3–54.4)	17.56 ± S.D. 1.82 (14.79–21.73)	1634 ± S.D. 172 (1360–2060)
Di Dii	1954–55 1955–56	9	24.4 (19–35)	177 (161–186)	69.9 (55.2–78.9)	3062 ± S.D. 327 (2340–3449)	43.9 ± S.D. 3.56 (39.3–50.4)	17.30 ± S.D. 1.37 (14.10–18.78)	1656 ± S.D. 116 (1421–1792)
E	1955–56	18	23.2 (20–26)	176 (166–184)	72.8 (65.3–84.3)	3129 ± S.D. 262 (2661–3552)	43.2 ± S.D. 4.48 (34.7–50.2)	17.75 ± S.D. 1.15 (15.49–19.39)	1671 ± S.D. 122 (1449–1872)
	Means Range	72	23.1 (19–35)	176 (161–189)	73.9 (55.2–92.9)	3103 ± S.D. 290 (2340–4041)	42.4 ± S.D. 4.74 (31.3–54.4)	17.56 ± S.D. 1.38 (14.10–21.73)	1645 ± S.D. 130 (1360–2060)

NOTE: The groups studied over 3 years were fairly comparable in age, height, and weight. The mean plasma volumes, along with standard deviations and ranges, are shown as absolute volume and also in terms of height, weight, and surface area. These figures are in general agreement with reports in the literature.

being exposed to room temperatures of 20–25° C. for the greater part of each day. During the summer months they were employed in a variety of occupations as laboratory workers, sleeping car porters, light manual workers, etc.

It will be seen from Table I that groups comprising the total of 72 subjects used throughout the 3 years of the study were fairly comparable in age, height, weight, and mean plasma volume. The plasma volumes shown in this table are in general agreement with published reports for adult male subjects (12, 20, 22). There was considerable individual variation in weight fluctuation with a tendency for the weight to be about 1 kg. lower in summer than in winter, but with no difference between the beginning and the end of the study periods, which was in April. Most of the subjects were studied serially over a 12-month period. However, one group of nine (Group D) was studied for 2 consecutive years. The 13 subjects of Group B were each given 400 mg. ascorbic acid orally daily from May 1953 to December 1953, and the 18 subjects of Group A were given this supplement from December 1953 to April 1954 (18).

Experimental Procedure

Serial studies were made at regular intervals throughout the 12-month study periods—for Groups A, B, E, and Dii at 4-weekly intervals, and for Groups C and Di at 8-weekly intervals.

The subjects attended in the morning without breakfast; their height and weight were recorded. They then rested on a cot for 30 minutes before a 20-ml. venous blood sample was taken and a carefully-measured quantity of a 0.2% solution of Evans blue dye (T1824) was injected intravenously. Ten minutes later a 10-ml. venous sample was withdrawn from the opposite arm. In addition to the plasma volume estimation, the haemoglobin concentration and haematocrit were measured on each occasion. During the last 2 years of the study plasma protein concentration was also measured. Of the 23 subjects of Groups C and Di, eight had serial estimations of total body water and extracellular fluid space.

During the last 2 years of the study care was taken to maintain the temperature of the laboratory during all the estimations at 20° ± 1° C. and the relative humidity between 40 and 50%.

Analytical Procedures

The calculation of plasma volume from the serum concentration of T1824 was, essentially, according to the method of Gibson and Evans (12). The analytical procedure was modified somewhat to achieve greater accuracy. The optical density of the serum of the 10-minute sample of blood, carefully separated to avoid hemolysis, was measured in a Beckman DU quartz spectrophotometer at wave length 620 mμ and compared with that of a freshly prepared standard made by adding with a Kirk microtransfer pipette, 0.005 ml. of the same dye as used for injection to 2.5 ml. of serum from the blood sample taken just before the dye injection. By this means the error introduced by differing optical densities of sera is minimized.

The dye was injected in syringes calibrated by weighing to ensure greater accuracy. In most of the estimations 10 ml. of dye solution was used but in some smaller subjects 5 ml. was found to give adequate serum levels.

Validity of the Method

The validity of using a single 10-minute sample to measure dye dilution has been discussed by several authors (14, 23, 25). Rossiter (25) found that in subjects with low plasma proteins a considerable error may be avoided by taking repeated samples to establish the slope of the disappearance curves. Although our subjects were healthy, with plasma proteins within the normal range, the available data have been analyzed to determine if a varying disappearance slope would explain, to some extent, the seasonal fluctuations in plasma volume calculated from the 10-minute sample. It is also possible that loading the body with repeated injections of dye at monthly intervals would influence the disappearance curve and, therefore, the calculated blood volume.

Dye disappearance curves with blood samples taken at 10 minutes, and 1, 2, and 3 hours after injection were studied on 44 occasions in 11 subjects.

The slopes of the log dye concentration plotted against time were not significantly different between the summer and winter, nor was there any evidence that previous dye injections influenced the 3-hour disappearance; also the disappearance slope was of the same order in all 11 subjects.

When the plasma volume calculated from the 10-minute sample was compared to that calculated from the disappearance curve extrapolated to zero time, the results were in close agreement but with no constant relationship between them. In the 44 comparisons the range of variation of the 10-minute sample, expressed as a per cent of the extrapolated value, was from 2.5% above to 3.0% below with a mean difference of only 0.2%.

From repeated tests on the same subjects at intervals of 2 to 3 days, von Porat (22) concluded that the error of the method was approximately 5%. We have not repeated such a test of precision of the method. However, some index of the precision of the results of the groups is given by the standard errors of the monthly means (Fig. 1). These were usually less than 2% of the mean, which is considered sufficiently small to allow study of changes of the magnitude expected from seasonal variations (3).

Toxicity of T1824

In keeping with most reports of both animal and human administration (22) and in contrast with the case report of Roberts (24), no serious adverse side effects were encountered. The nine subjects of Group D (Table I) had the largest total dosage of dye. This ranged between 220 mg. to 310 mg. spread over 2 years.

There were a few minor reactions. Three subjects in 1 week had a mild febrile reaction 1 to 1½ hours after injection but they had no further reactions on subsequent tests; three other subjects had transient flushing and tachycardia within a few minutes of the dye injection. In each case this was at the end of a series and the subjects had no further tests.

Approximately 20 tests were incomplete owing to extravascular dye injection. This caused no discomfort but produced a local blue discoloration which disappeared in 2 to 3 weeks.

Generalized slight blueness of the skin was noted in some subjects after a few injections, particularly in small fair subjects. On this account approximately 25% of the tests were carried out using 10 mg. of dye rather than the usual 20 mg.

Further investigation of this phenomenon was carried out by a study of the long-term disappearance of the dye from the blood. It was concluded that there was no progressive deterioration of the body's ability to remove the dye with repeated injections because the amount present in the serum 30 days after each injection ceased to rise after the third or fourth injection. The 3-hour disappearance curves mentioned in the preceding section were calculated from the optical density difference between the sample taken immediately before the injection and those taken in the succeeding 3 hours. Although the "blank" sample had an appreciable amount of dye, this did not influence the 3-hour disappearance curve of the injected dose.

Finally, the health of the subjects was unchanged by as many as 20 repeated injections, using as criteria the haemoglobin concentration, haematocrit, plasma protein concentration, weight, and sense of well-being. We therefore consider this method to be safe and innocuous.

Other Analytical Methods

Estimations of total body water and extracellular fluid space were made using an intravenous infusion containing 1 g. antipyrine and 8 g. inulin, made up to 100 ml. with normal saline. This was given over 10 to 20 minutes, starting when the 10-minute sample for T1824 estimation had been taken. Blood samples were taken at 1, 2, and 3 hours after the mid-point of the infusion and the serum concentration of antipyrine and of inulin at zero time were calculated by extrapolation. Antipyrine concentration in the injection fluid and in serum samples was determined by the method of Brodie (6); inulin concentration was measured by a modification of Roe's resorcinol method used by Bacon and Bell (1).

Haemoglobin was estimated by the cyan-haematin method (17), haematocrit by Wintrobe's method (32), and total plasma proteins by the biuret reaction method (13).

Results

Seasonal Fluctuations in Plasma Volume

For purposes of illustrating the main results, the individual plasma volume estimations for each subject were calculated as per cent of the mean of all determinations on that subject. This calculation is considered valid as the correlation between the mean plasma volumes and the maximal changes which occurred was found to be statistically significant. The derived data were then grouped and plotted as monthly means in Fig. 1, the vertical bars representing one standard error above and below the mean.

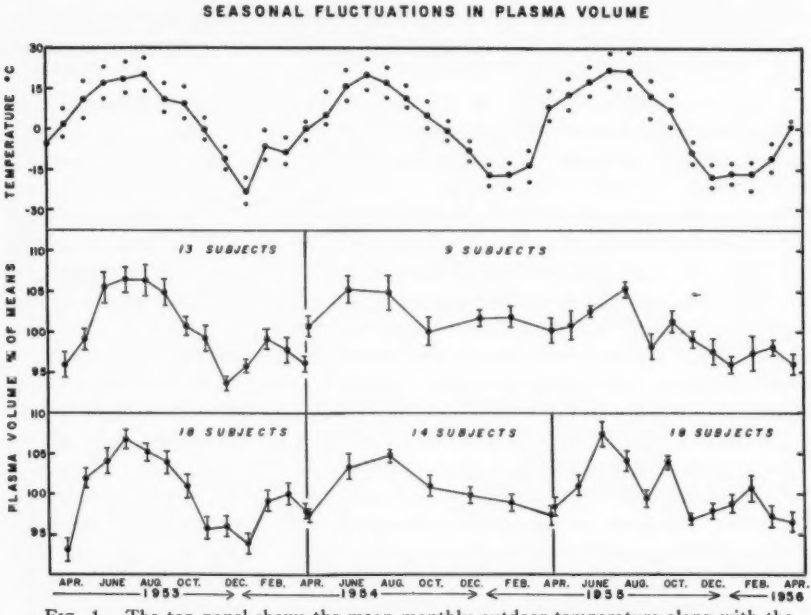


FIG. 1. The top panel shows the mean monthly outdoor temperature along with the mean daily maxima and minima. This data was obtained from the Winnipeg office of the Meteorological Division, Department of Transport, Canada. The lower panels summarize the plasma volume measurements of 72 subjects over 3 years, as described in the text.

With the data expressed in this way, the peaks and troughs for each group can be compared by the method of the standard error of the difference between means. The significance of the seasonal changes can also be assessed by analysis of paired data. Occasionally the latter method detected significant changes which were not apparent from a consideration of the group means. The probabilities noted below are based on analysis of paired data.

In the first year Groups A and B, considered separately or together, show an obvious and highly significant increase in plasma volume during the summer and a subsequent decline in the autumn ($p < .001$). The increase from December to February in that year was also a significant one in both Groups A and B ($p < .05$ and .01 respectively). There was no difference between Groups A and B that could be attributed to the ascorbic acid supplement.

The seasonal fluctuations were much less marked during the 2nd year. The changes in the nine subjects of Group Di were not statistically significant but in the 14 subjects of Group C the increase in the summer and the gradual decline throughout the winter were shown to be significant at the 5% level.

During the 3rd year of the study more significant fluctuations were again found. In Group Dii the decrease from summer to winter was significant at the 1% level and the change from July–August to September was also significant at the 5% level. In the 18 subjects of Group E the summer increase and

winter decrease were highly significant ($p < .001$). The data for October in this group are significantly higher than those for September or November. This last finding was unexpected and attempts were made to explain it by some unusual changes in the weather, or in the behavior of the subjects, or in the technique of the estimations, but in each case without success. The same trend can be seen in Group Dii.

Comparison from month to month does not illustrate a decrease in plasma volume after an initial increase associated with the change from a cool to a hot environment as found by Bazett *et al.* (4) and invoked by Brown *et al.* (7) as a possible explanation of the decrease in blood volume of Eskimos during the summer.

Variations in the Seasonal Pattern of Plasma Volume

In spite of the clear-cut changes in plasma volume shown in Fig. 1, there was considerable variation from subject to subject. This point is illustrated in Fig. 2, which compares two subjects of Group B who were studied simultaneously. Subject 7 had a much greater seasonal swing than subject 19. Both were large subjects, 80 and 90 kg. respectively, but with almost equal mean plasma volumes, 3200 and 3260 ml. respectively. Subject 19 had a plasma volume peak in November when that of subject 7 was approaching its lowest value.

As mentioned in the previous section, the secondary fluctuation in September, October, and November 1955 which affected both Groups Dii and E remains unexplained.

Seasonal Variations in Red Blood Cells and Plasma Proteins

The haemoglobin concentration and haematocrit showed only slight fluctuations and usually in the opposite direction to that of the plasma volume. Although these fluctuations were proportionately less than those in the plasma volume, they sometimes achieved statistical significance. The net result of these relatively small changes is that the total blood volume, total circulating haemoglobin, and red cell mass fluctuate seasonally in parallel fashion to the plasma volume.

When the haemoglobin and haematocrit are considered together as the mean corpuscular haemoglobin concentration (M.C.H.C.) an increase is seen during the summer months concomitant with the increase in plasma volume and, like the plasma volume, it is least noticeable in 1954–55 of the 3 years of the study (Fig. 3). The increases in M.C.H.C. in the summers of 1953 and 1955 and the subsequent decrease in 1955–56 are statistically significant at the 1% level or less.

Although the over-all mean of the calculated M.C.H.C. is consistent with reports of normal values (32), the summer means are high and several individual readings (based on duplicate analysis of both haemoglobin and haematocrit on blood samples collected with a minimum of stasis) were well above the range usually accepted for normals.

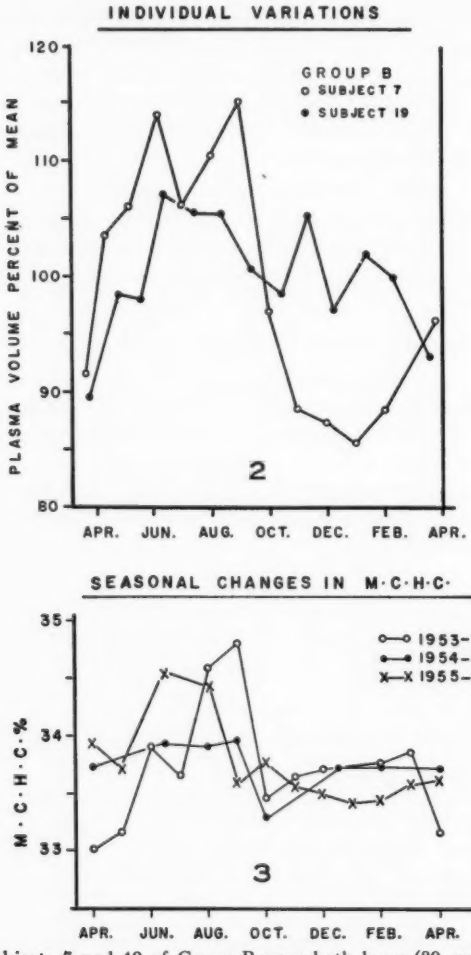


FIG. 2. Subjects 7 and 19 of Group B were both large (80 and 90 kg.) and with similar mean plasma volumes (3200 and 3260 ml.). However, the plasma volume pattern differs not only in the degree of change above and below the mean but in the time of their respective maxima and minima.

FIG. 3. There is a significant increase in mean corpuscular haemoglobin concentration during the summer months in 2 of the 3 years of the study. Like the plasma volume the change is least during 1954.

Relation Between Total Body Water, Extracellular Space, and Plasma Volume

In eight subjects of Groups C and Di, inulin space and antipyrine space data are available concomitant with the plasma volume, and in four additional subjects plasma volume and antipyrine space were measured twice, in January and July 1956. These data are summarized as means in Table II where all results are expressed in terms of liters per sq. m. body surface. The mean total

TABLE II

BODY FLUID COMPARTMENTS

Liters per sq. m. of body surface

	June–Aug.	October	Dec.–Jan.	Feb.–March	April
	Eight subjects 1954–55				
Total body water	20.83 ±1.90*	21.20 ±1.85	20.88 ±1.72	20.87 ±1.96	21.15 ±1.97
Extracellular space	7.56 ±0.56	7.24 ±0.69	7.33 ±0.71	7.74 ±0.64	7.65 ±0.85
Plasma volume	1.72 ±0.21	1.65 ±0.24	1.68 ±0.21	1.67 ±0.19	1.68 ±0.25
	Four subjects 1956				
Total body water	22.68	—	22.08	—	—
Plasma volume	1.76	—	1.60	—	—

NOTE: A comparison of total body water (antipyrine space), extracellular space (inulin space), and plasma volume (T1824 space) in 12 subjects.
*Standard deviation of mean.

body water values for individuals varied from 17.56 to 24.50 with an average mean of 20.94 liters per sq. m. These findings agree with those of other workers (5, 28, 30, 21, 10, 15, 9, 16, 26, 31). The range of variation was less in repeated estimations on the same subject (the coefficient of variation for all eight subjects ranged from 0.8 to 4.4%) than the variations between subjects tested simultaneously (coefficient of variation approximately 9%). The same remarks apply to the extracellular space measured as inulin space and expressed as liters per square meter body surface.

No consistent relationship was seen between the comparatively small fluctuations in total body water from time to time and the plasma volume changes or between the inulin space and plasma volume. However, little change in plasma volume was experienced that year so that no conclusions could be arrived at, except that the total body water measurement was quite reproducible. On this account four more students were investigated by the simultaneous determination of total body water and plasma volume, once during the winter and again in the summer. In each case the plasma volume increased; the average for the four subjects was approximately 10%. Although the repeated estimations on the same subject were not as close as the previous experiments with eight subjects, there was again no relationship between variations in total body water and those in plasma volume.

Discussion

The results of the plasma volume determinations indicate that there is a considerable increase in blood volume in healthy young males in Winnipeg in the summer and that this trend is reversed in the fall and winter.

Although no evidence is presented on which to base an opinion concerning the cause, let alone the mechanism, of this cyclic alteration, it is clear that not only is there considerable difference between individuals but that the extent of the variation may vary from year to year. No doubt muscular activity may play a modifying role on plasma volume (23) and possibly hours of daylight may have some influence (27), but it seems highly likely that the environmental temperature plays an important role, directly or indirectly. LeBlanc *et al.* (18) have found that ascorbic acid supplement increased the tolerance of humans to a regime of cold and food deprivation. Therefore it seemed of interest to ascertain if this vitamin would affect the blood volume. There is no suggestion from the results that ascorbic acid affects this aspect of acclimatization. The decrease in plasma volume after the initial increase, which was found by Brown (7), was not apparent, perhaps owing to very different environmental exposure of the students used in the present study from the subjects employed in his.

The fact that these changes have been found implies a seasonal acclimatization in Winnipeg under ordinary sedentary indoor conditions. However, the variations in the pattern found indicates that such seasonal evidence of acclimatization cannot be relied on to occur invariably in normal subjects in these circumstances.

Although the data reported here do not allow clear-cut conclusions to be drawn, it is suggested that the seasonal fluctuations found are limited to the vascular system and do not affect the other fluid compartments. If this is indeed the case, then the mechanism of the plasma volume changes may be found in the vascular system.

An interesting corollary of the cyclic change in total blood volume is that there must be either a seasonal change in erythropoietic activity or in the destruction of erythrocytes. Neither of these possibilities has been investigated in the present study.

References

1. Bacon, J. S. D. and Bell, D. J. Biochem. J. **42**, 397 (1948).
2. Barcroft, J., Binger, C. A., Bock, A. V., Doggert, J. H., Forbes, H. S., Harrop, G., Meakins, J. C., and Redfield, A. C. Trans. Roy. Soc. (London), B, **211**, 351 (1922).
3. Bass, D. E. and Henschel, A. Physiol. Revs. **36**, 128 (1956).
4. Bazett, H. C., Sunderman, F. W., Doupe, J., and Scott, J. C. Am. J. Physiol. **129**, 69 (1940).
5. Behnke, A. R., Feen, B. G., and Welham, W. C. J. Am. Med. Assoc. **118**, 495 (1942).
6. Brodie, B. B. Methods in medical research. Vol. 4. Year Book Publishers Inc., Chicago. 1955. p. 31.
7. Brown, C. M., Bird, G. S., Boag, L. M., Delahaye, D. J., Green, J. E., Hatcher, J. D., and Page, J. Metabolism, **3**, 247 (1954).
8. Conley, C. L. and Nickerson, J. L. Am. J. Physiol. **143**, 373 (1945).
9. Edelman, I. S., Haley, H. B., Schloerb, P. R., Sheldon, D. B., Friis Hansen, B. J., Stoll, G., and Moore, F. D. Surg. Gynecol. Obstet. **95**, 1 (1952).
10. Faller, I. A., Petty, D., Last, J. H., Pascale, L. R., and Bond, E. E. J. Lab. Clin. Med. **45**, 748 (1955).
11. Forbes, W. H., Dill, D. B., and Hall, F. G. Am. J. Physiol. **130**, 739 (1940).
12. Gibson, J. G. and Evans, W. A. J. Clin. Invest. **16**, 317 (1937).
13. Gornall, A. G., Bardawill, C. J., and David, M. M. J. Biol. Chem. **177**, 751 (1949).
14. Gregersen, M. I. J. Lab. Clin. Med. **29**, 1266 (1944).
15. Hardy, J. D., Sen, P. K., and Drabkin, D. L. Surg. Gynecol. Obstet. **93**, 103 (1951).

16. IKKOS, D., LUFT, R., SJÖGREN, B. Metabolism, **3**, 400 (1954).
17. KING, E. J. and GILCHRIST, M. Lancet, **2**, 201 (1947).
18. LEBLANC, J., STEWART, M., MARIER, G., and WHILLANS, M. G. Can. J. Biochem. Physiol. **32**, 407 (1954).
19. MAXFIELD, M. E., BAZETT, H. C., and CHAMBERS, C. C. Am. J. Physiol. **133**, 128 (1941).
20. NOBLE, R. L. and GREGERSEN, M. I. J. Clin. Invest. **25**, 158 (1946).
21. OSSERMAN, E. F., PITTS, G. C., WELHAM, W. C., and BEHNKE, A. R. J. Appl. Physiol. **2**, 633 (1950).
22. VON PORAT, B. T. D. Acta Med. Scand. **140**, Suppl. 256 (1951).
23. REEVE, E. B. Nutrition Abstr. & Revs. **17**, 811 (1948).
24. ROBERTS, L. N. Can. Med. Assoc. J. **71**, 489 (1954).
25. ROSSITER, R. J. Lancet, **1**, 222 (1949).
26. SIRI, W. E., REYNAFARJE, C., BERLIN, N. I., and LAWRENCE, J. H. J. Appl. Physiol. **7**, 333 (1954).
27. SJÖSTRAND, T. Physiol. Revs. **33**, 202 (1953).
28. SOBERMAN, R., BRODIE, B. B., LEVY, B. B., AXELROD, J., HOLLANDER, V., and STEELE, J. M. J. Biol. Chem. **179**, 31 (1949).
29. SPEALMAN, C. R., NEWTON, M., and POST, R. L. Am. J. Physiol. **150**, 628 (1947).
30. STEELE, J. M., BERGER, E. V., DUNNING, M. F., and BRODIE, B. B. Am. J. Physiol. **162**, 313 (1950).
31. TENG, H. C., SHAPIRO, A. P., and GROLLMAN, A. Metabolism, **3**, 405 (1954).
32. WINTROBE, M. M. Clinical hematology. 3rd ed. Lea & Febiger, Philadelphia. 1951.

THE EFFECT OF BZ-55 (CARBUTAMIDE) ON THE GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE LIVERS OF INTACT AND ALLOXANIZED FEMALE RATS[1]

R. D. Hawkins and R. E. Haist

Abstract

The ingestion of the sulphonylurea BZ-55 (0.5 g./kg. body weight) by normal female rats for 8 days leads to a significant depression in the level of glucose-6-phosphatase activity in the liver of these animals. The glucose-6-phosphatase activity of the liver in alloxanized rats is higher than that of paired-fed control animals. The ingestion of BZ-55 by alloxanized rats causes this activity to be further increased as shown by comparison with the glucose-6-phosphatase activity in alloxanized, individually paired-fed controls. The differences in the effects of BZ-55 in normal and alloxanized animals are discussed.

Recent investigations have shown that in the livers of rats maintained on a chow diet to which the sulphonylurea BZ-55[2] (N-sulphanilyl-N′-butyl carbamide) was added, the glucose-6-phosphatase activity was significantly less than in those of intact, paired-fed control animals (6). The experiments to be reported here were done to determine whether or not a similar effect could be elicited in animals in which the number of functioning *beta* cells in the islets of Langerhans had been reduced to an extremely low level by the intravenous administration of alloxan.

Materials and Methods

Female rats of the Carworth strain, weighing 125–135 g., were put in individual cages and weighed daily. They were fed a diet of ground chow and divided into four groups.

Group 1 consisted of eight normal rats individually paired-fed with members of Group 2. These served as controls.

Group 2 consisted of eight rats fed ground chow containing BZ-55. The intake of BZ-55 was 0.5 g. per kg. body weight daily for 8 days.

Groups 3 and 4 were made up of alloxanized rats. Thirty-four rats received intravenously 70 mg. alloxan per kg. body weight. Starting on the 2nd day following the alloxan injections these rats received 0.5–1.0 unit of protamine-zinc insulin per day for 2 to 3 days until their initial body weight was regained. Insulin was then stopped 2 days before the experiment started, to allow the circulating insulin to be reduced to low levels. On the basis of the severity of the diabetes, as judged by the extent of polyuria and glycosuria, 24 of these animals were divided into two comparable groups. Group 3 consisted of 12 control alloxanized animals. These were individually paired-fed with members of Group 4. Group 4 consisted of 12 alloxanized rats receiving chow containing BZ-55. For a period of 8 days these rats ingested 0.5 g.

[1]Manuscript received November 19, 1956.
Contribution from the Department of Physiology, Charles H. Best Institute, University of Toronto, Toronto, Ontario. This work was aided by a grant from the National Research Council of Canada.

[2]BZ-55 was kindly supplied by Eli Lilly and Co., Indianapolis, Indiana, U.S.A.

of BZ-55 per kg. body weight daily. Of the 12 pairs of alloxanized animals in Groups 3 and 4, eight pairs were kept in fairly good condition for 8 days, but the other four pairs had to be sacrificed earlier.

The animals were killed with an overdose of ether and the liver was immediately removed and weighed. A weighed aliquot of liver was fast-frozen, to be homogenized later and used for the estimation of enzyme activity.

Glucose-6-phosphatase activity of the freshly prepared homogenate of rat liver was measured according to the method of Cori and Cori (4). One gram of tissue was homogenized with 9 ml. ice-cold distilled water in a Potter–Elvehjem homogenizer and a measured aliquot, equivalent to 20 mg. of fresh liver, was incubated at a temperature of 30° C. in the presence of tris(hydroxymethyl)aminomethane buffer (0.05 *M*, pH 6.6) with the potassium salt of glucose-6-phosphate in a final concentration of 0.1 *M*. The potassium salt of glucose-6-phosphate was prepared from the barium salt by mixing with the latter its stoichiometric equivalent of potassium sulphate, shaking, and then centrifuging to remove the insoluble precipitate. Maximal activity is obtained with 0.1 *M* substrate concentration. This activity is higher than that reported in our previous experiments, where the substrate concentration was 0.005 *M* (6). Appropriate tissue and substrate controls were run concomitantly to detect the presence of inorganic phosphate in the preparations. Inorganic phosphate was measured by the method of Fiske and SubbaRow (5).

Experimental Results

In Fig. 1 the changes in mean body weight for the four groups are shown. The alloxanized animals lost weight initially, but BZ-55 had little effect on body weight during the experimental period. The same applies to the effect of BZ-55 in the non-alloxanized animals.

Fig. 2 indicates the mean glucose-6-phosphatase activity for each of the four groups, expressed as activity per gram of fresh liver and total hepatic activity. Statistical analyses showed that the differences between the means

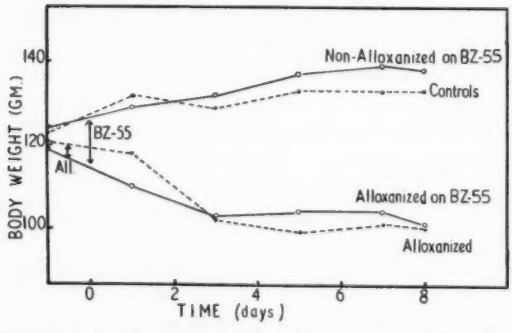


FIG. 1. Changes in body weight in alloxanized and non-alloxanized rats, with and without the addition of BZ-55 to the diet.

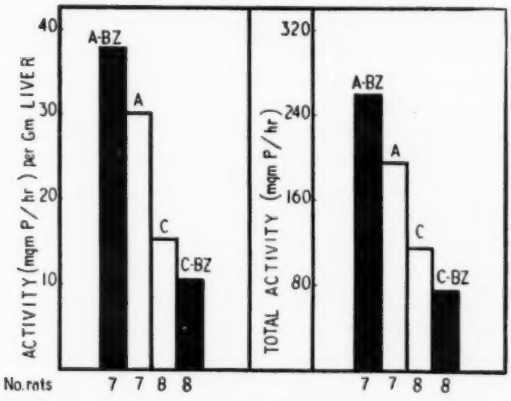


FIG. 2. Mean glucose-6-phosphatase activities in the livers of non-alloxanized and alloxanized rats, with and without the addition of BZ-55 to the diet. Enzyme activity was measured at 30° C. and allowed to proceed for 20 minutes. Reaction vessels contained 0.3 ml. buffer, pH 6.6, 0.2 ml. freshly prepared homogenate equivalent to 20 mg. wet wt. liver, 0.5 ml. potassium salt of glucose-6-phosphate (0.2 *M*). The reaction was stopped by the addition of 1 ml. trichloroacetic acid (10%), and the inorganic phosphate was measured by the method of Fiske and SubbaRow (5). Appropriate tissue and substrate controls were run at the same time. Activity was calculated on the basis of mg. phosphorus released per hr. per g. wet weight of liver or per total wet weight of liver. C = non-alloxanized control rats; C-BZ = non-alloxanized rats receiving BZ-55; A = alloxanized animals; A-BZ = alloxanized animals receiving BZ-55.

are significant at the 1% level. It will be seen that the ingestion of BZ-55 for 8 days causes a depression in glucose-6-phosphatase activity in the non-alloxanized animal, both in the concentration of enzyme per unit wet weight of tissue and in total hepatic activity. Treatment with alloxan results in a rise in glucose-6-phosphatase activity of the liver, a finding which has been reported previously by Ashmore and his associates (1, 2) and by Langdon and Weakley (7). In the alloxanized animal the ingestion of BZ-55 does not cause a drop in enzyme activity, but instead induces a significant increase, both in the concentration of enzyme and in its total activity in the liver.

Discussion

It has been demonstrated by Ashmore and his co-workers (2) that the injection of insulin causes a decrease in the glucose-6-phosphatase activity of the liver in the intact rat, and Langdon and Weakley (7) have shown that the injection of protamine-zinc insulin into alloxanized rats 12 hours prior to sacrifice brings the elevated enzyme level down to within the normal range (7). It is possible that, in the present experiments, the decrease in glucose-6-phosphatase activity which was observed following the ingestion of BZ-55 by intact animals is an indirect effect of an increased secretion of insulin by the *beta* cells of the islets. The suggestion that the islets are stimulated receives support from the fact that Ashworth and Haist (3) have recently shown that the administration of BZ-55 per os to rats for 3 weeks leads to a significant increase in the volume of the islets of Langerhans. It

might be expected that if insulin secretion is normally a factor, then when a severe depletion in functioning *beta* cells occurs there would be an increase in glucose-6-phosphatase activity in the liver. Such a finding is obtained when the animals are made diabetic by alloxan. The ingestion of BZ-55 by alloxanized rats would be expected to elicit little change in enzyme activity if the effect of BZ-55 on enzyme activity were due solely to stimulation of insulin secretion, or if the presence of insulin is required for the action of this compound. The present results indicate that there is not merely an absence of a fall in the glucose-6-phosphatase activity of liver in the alloxanized rats treated with BZ-55, but actually there is a further increase in the activity of the enzyme in the livers of this group. This may be due to effects of BZ-55 not mediated by insulin, the absence of insulin altering or unmasking the effects of BZ-55 on other tissues, or to a still further reduction in available insulin after treatment with BZ-55 in the alloxanized animal. It should be pointed out that these effects of BZ-55 on glucose-6-phosphatase were obtained after a period of treatment and were not immediate effects. It seems likely that the effect is related to changes in the amount of the enzyme present, rather than to an interference with its action.

References

1. ASHMORE, J., HASTINGS, A. B., and NESBETT, F. B. Proc. Natl. Acad. Sci. U.S. **40**, 673 (1954).
2. ASHMORE, J., HASTINGS, A. B., NESBETT, F. B., and RENOLD, A. E. J. Biol. Chem. **218**, 77 (1956).
3. ASHWORTH, M. A. and HAIST, R. E. Can. Med. Assoc. J. **74**, 975 (1956).
4. CORI, G. T. and CORI, C. F. J. Biol. Chem. **199**, 661 (1952).
5. FISKE, C. H. and SUBBAROW, Y. J. Biol. Chem. **66**, 375 (1925).
6. HAWKINS, R. D., ASHWORTH, M. A., and HAIST, R. E. Can. Med. Assoc. J. **74**, 972 (1956).
7. LANGDON, R. G. and WEAKLEY, D. R. J. Biol. Chem. **214**, 167 (1955).

BIOSYNTHESIS OF QUERCETIN IN BUCKWHEAT

PART I[1]

E. W. UNDERHILL,[2] J. E. WATKIN,[3] AND A. C. NEISH

Abstract

Cuttings of *Fagopyrum tataricum* were allowed to metabolize various labelled compounds for 24 hours in the light. Quercetin was then isolated and degraded by alkaline hydrolysis of its pentamethyl ether into veratric acid (ring B plus carbon 2) and 2′-hydroxy-2,4′,6′-trimethoxyacetophenone (ring A plus carbons 3 and 4). Eleven amino acids, including tyrosine, were poor precursors of quercetin as were also protocatechuic acid and *p*-hydroxybenzoic acid. Shikimic acid, phenylalanine, *p*-hydroxycinnamic acid, and cinnamic acid were very good precursors followed by (in decreasing order of effectiveness) caffeic acid (fair), sinapic acid, *m*-methoxycinnamic acid, and ferulic acid (very poor). Quercetin formed from β- or ring-labelled cinnamic acid gave labelled veratric acid on degradation, and that from α- or carboxyl-labelled cinnamic acid gave labelled 2′-hydroxy-2,4′,6′-trimethoxyacetophenone. Two-ninths of the activity of quercetin formed from uniformly labelled phenylalanine was found in the acetophenone derivative and seven-ninths in the veratric acid. These results show that $C_6.C_3$ compounds are used with the carbon skeleton unchanged for the synthesis of ring B and carbons 2, 3, and 4 of quercetin and that the ring hydroxylation pattern of the compound used determines its effectiveness. None of the compounds were good precursors of ring A.

Introduction

The literature on the occurrence of flavonoid pigments in nature and their structural relations has been covered in several excellent reviews (10, 16, 19). A number of theories on the biosynthesis of flavones and anthocyanins have been proposed. These theories are based chiefly on the structural relationships (i.e. the hydroxylation patterns) between flavonoids found in the same plant. There has been little work, using biochemical techniques, to test the validity of these hypotheses. However Birch, Donovan, and Moewus (1) have carried out experiments with the unicellular green alga *Chlamydomonas eugametos* which suggest a plausible biochemical pathway for formation of the ubiquitous flavone, quercetin (I, Fig. 1). These workers obtained 26 mutant female strains of the alga, all of which produced gametes which would copulate with those of a male strain if quercetin was added. Substitution experiments with a number of possible precursors of quercetin, using the copulation test, indicated that the alga formed quercetin through a pathway involving condensation of caffeic acid and phloroglucinol. The phloroglucinol was derived from *meso*-inositol and the caffeic acid from phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine, and 3,4-dihydroxyphenylpropionic acid.

[1]Manuscript received October 9, 1956.
Contribution from the Prairie Regional Laboratory, National Research Council, Saskatoon, Sask.
Issued as N.R.C. No. 4210.
[2]Graduate Student assisted by National Research Council Studentship, College of Pharmacy, University of Saskatchewan, Saskatoon, Sask.
[3]Postdoctorate Fellow, National Research Council of Canada 1955–1956.

In the present paper experiments are reported which use another approach to the problem of biosynthesis of quercetin. Buckwheat plants were selected as the experimental material since they are readily grown and contain rutin, a rhamnoglucoside of quercetin, in the leaves in yields which may exceed 5% of the dry weight (11, 15, 20). A number of C^{14}-labelled compounds were administered, in approximately equal amounts, to buckwheat tops harvested at a stage of growth when rutin was being rapidly synthesized. The relative effectiveness of these compounds as precursors was assessed by measuring the specific activity of the quercetin isolated after a fixed period of metabolism. Active samples of quercetin were degraded (Fig. 1) to measure the distribution of C^{14} in the molecule.

Experimental

General Techniques

Radioactivity measurements were made by the sensitive gas-phase proportional counting technique of Buchanan and Nakao (5). This method involves combustion of the compound to carbon dioxide, which is measured manometrically and then mixed with methane in a gas counter. During 3 years of use in this laboratory the method has been found to be quite reliable. The melting points (corrected) were determined with a Kofler hot-stage microscope. Unless stated otherwise, evaporations of small volumes of solutions were carried out by blowing a stream of dry, filtered air onto the surface of the solution, contained in a beaker at room temperature.

C^{14}-Labelled Compounds

The labelled phenylacetic acid and all of the labelled amino acids were purchased from Atomic Energy of Canada. Shikimic acid-C^{14} and the labelled aromatic acids were prepared as described previously (2, 3, 4).

Cultivation of Plants

The buckwheat seeds (*Fagopyrum tataricum* Gaertn. var. C.D. 4251) were a gift from Dr. W. G. McGregor, Cereal Division, Central Experimental Farm, Ottawa. The seeds were planted directly in a gravel bed which was irrigated automatically from below (8) with a modified Hoagland solution (14). The greenhouse temperature was controlled at approximately 20° C. during the winter months and supplementary illumination was provided by tungsten and fluorescent lights to give a day length of 18 hours with an intensity of about 1000 ft-c.

The rutin content of buckwheat leaves increases with age to a maximum at the time of flowering and then decreases (6, 20). The plants used for tracer experiments were taken at the time of appearance of the first flower buds (4 to 5 weeks after seeding) when rutin synthesis was known to be rapid.

Administration of C^{14}-Labelled Compounds to Plants

Four to six tops were used in each experiment. The stems were cut under water with a razor blade to remove the roots and the cuttings were then placed in a small beaker containing the C^{14}-labelled compound dissolved

in 1 to 2 ml. of water. The aromatic acids were administered as their sodium salts. The illuminated plants absorbed the solution within ½ hour; the last traces were washed into the plant by addition of three successive 1-ml. portions of water to the beaker as it approached dryness. The cuttings were then transferred to a beaker containing about 100 ml. of tap water and allowed to metabolize in an illuminated incubator (400 ft-c.) at 20–21° C. for 24 hours.

Isolation and Purification of Quercetin

The stalks were sliced both longitudinally and transversely and the plant material was dried in a circulating oven at 60° C. overnight. It was then ground to pass the 40 mesh screen of a semimicro Wiley mill. The ground material (5 g.) was extracted by two successive digestions with 200 ml. portions of boiling 80% ethanol, each for 5 minutes, and the residue was washed on the filter with a further 100 ml. of hot 80% ethanol.

The combined filtrate was evaporated to about 50 ml. under reduced pressure, filtered with celite, and the filtrate extracted twice with an equal volume of ethyl ether. The aqueous phase was evaporated to about 10 ml. and then cooled overnight in a refrigerator. The crystalline rutin was separated by filtration, washed with cold water, and, after being sucked dry, with ether. This crude rutin was hydrolyzed by refluxing as a 0.5% slurry in *N* sulphuric acid. The rutin dissolved after a few minutes' boiling and then quercetin crystallized out as the hydrolysis was continued. After 1½ hours' heating the mixture was cooled and the quercetin was isolated by filtration and washed with water.

This crude quercetin was purified by chromatography, as follows. Magnesol* was dried in a vacuum oven at 80° C. for 5 hours. It was slurried in petrol (boiling range, 75–90° C.) and made into a column 20 × 40 mm. The quercetin (which is insoluble in petrol) was dissolved in 2 to 3 ml. of hot acetone, 3 ml. of hot benzene added, and the mixture immediately poured on the column. The quercetin was adsorbed as a sharp narrow band at the top of the column. If benzene was omitted the quercetin was adsorbed as a wide diffuse band. After adsorption of the quercetin the acetone and benzene were washed out of the column with petrol. The column was then developed with water-saturated ethyl acetate. This removed quercetin completely but left the unreacted rutin and a red pigment adsorbed on the column. The quercetin moved down the column in association with an impurity which gave a brown fluorescence in ultraviolet light.

Further purification of the quercetin was effected by crystallization as the pentaacetate (9). The eluate from the magnesol column was evaporated, the residue (40–50 mg.) dried at 105° C. and dissolved in 1 ml. of dry pyridine. Acetic anhydride (1 ml.) was added and the solution allowed to stand at 0° C. for 5 hours. It was then poured into 20 ml. of ice water and stirred for 15 minutes. The crystalline pentaacetate was isolated by filtration and washed with cold water. It was dissolved in 3–5 ml. of hot acetone, 25 ml. of hot

*Magnesol is a synthetic hydrated magnesium silicate. It was obtained from the Westvaco Chlorine Products Co., South Charleston, West Virginia.

ethanol was added, and the mixture filtered. On cooling the filtrate under an air stream, quercetin pentaacetate crystallized out. Two recrystallizations carried out in this manner gave pure quercetin pentaacetate, m.p. 192–194° C.; yield 85–90% based on the quercetin.

Degradation of Quercetin

The alkaline cleavage of quercetin pentamethyl ether (12, 17), to veratric acid (III) and 2′-hydroxy-2,4′,6′-trimethoxyacetophenone (II), was utilized to obtain a measurement of the distribution of C^{14} between these two parts of the flavonol molecule (see Fig. 1).

The methylation procedure adopted, after considerable experimentation, was a modification of that used by Rao and Seshadri (18) for methylation of gossypitrin acetate. The radioactive sample of quercetin pentaacetate was diluted with enough unlabelled pentaacetate to give 200 mg. This was dissolved in 10 ml. of acetone in a 50 ml. Erlenmeyer flask. This solution was stirred throughout the methylation and the flask was cooled in a water bath to prevent boiling. Freshly distilled dimethyl sulphate (3 ml.) and 20% (w/v) aqueous sodium hydroxide (3 ml.) were added all at once, giving two liquid phases. After 2–3 hours, when the mixture became homogeneous, sodium hydroxide (3 ml.) was added in one portion. Two phases were again formed and after they became one phase (about 30 minutes) 2 ml. of dimethyl sulphate was added. After this mixture reverted to one phase 3 ml. of sodium hydroxide and 2 ml. of dimethyl sulphate were added in the same manner. Finally the mixture was made strongly alkaline using 10 ml. of sodium hydroxide, and stirred overnight. The acetone was evaporated; colorless crystals of quercetin pentamethyl ether separated rapidly. These were washed with cold water, dried, and recrystallized from benzene (5 ml.); yield 85–90%, m.p. 150–151° C.

The alkaline hydrolysis of quercetin pentamethyl ether (120 mg.) was carried out in a sealed stainless steel tube of 10 ml. capacity, containing 6.5 ml. of 12% (w/v) potassium hydroxide in 80% ethanol. After it was heated 1 hour in a boiling water bath the alkaline solution was transferred to a beaker with 20 ml. of water, made slightly acid with hydrochloric acid, and evaporated to about 15 ml. to remove the ethanol. The solution was neutralized with sodium bicarbonate and allowed to stand overnight in the refrigerator. The crystals of 2′-hydroxy-2,4′,6′-trimethoxyacetophenone which separated were filtered out and washed with cold water. The filtrate was extracted continuously with ether for 5 hours. The ether extract was discarded and the aqueous solution was acidified with hydrochloric acid (to pH 2 or lower) and again extracted with ether to remove the veratric acid.

A chromatographic column (20 × 60 mm.) was prepared from a mixture of equal parts (w/w) of Darco-G-60 charcoal and celite 535 slurried in ethanol. The 2′-hydroxy-2,4′,6′-trimethoxyacetophenone, dissolved in 3 ml. of ethanol, was added to the column, washed in with ethanol, and then eluted by a mixture of equal volumes of ethanol and benzene. The eluate was evaporated and the residue dissolved in 5 ml. of ethanol. Water (10 ml.) was added

and the volume reduced to 10 ml. by evaporation. This solution was cooled overnight at 0° C. The crystalline 2′-hydroxy-2,4′,6′-trimethoxyacetophenone was washed with cold water and dried in a vacuum desiccator; m.p. 102–104° C.; yield 70–75% based on quercetin pentamethyl ether.

The crude veratric acid, obtained by evaporation of the ether extract, was dissolved in 15 ml. of 2% aqueous sodium hydroxide. This solution was treated with 100 mg. of Darco-G-60 charcoal, heated, filtered, and the filtrate acidified with hydrochloric acid. Veratric acid crystallized out overnight at 0° C. and was filtered off and washed with cold water; yield 60–70%, m.p. 180–181° C.

Results

The relative effectiveness of various compounds as precursors of quercetin can be seen by comparing the dilution values given in the last column of Table I. Shikimic acid and phenylalanine, which gave quercetin showing the least dilution of C^{14}, were the best precursors of quercetin of all the compounds tested. Cinnamic acid and *p*-hydroxycinnamic acid were also good precursors but further substitution of hydroxyl and methoxyl groups in the aromatic ring of cinnamic acid gave compounds which were relatively poor precursors (i.e. caffeic, sinapic, and ferulic acids). The simpler aromatic acids (phenylacetic, protocatechuic, and *p*-hydroxybenzoic acids) were also poor precursors.

TABLE I

COMPARISON OF C^{14}-LABELLED COMPOUNDS AS PRECURSORS OF QUERCETIN IN BUCKWHEAT

Batch No.	Compound administered	Amount administered per g. dry wt. of plant		Specific activity (mμc./mM.)		Dilution*
		mμc.	μM.	Compound administered	Quercetin†	
1	Shikimic acid‡	1,040	43.0	24,200	676	36
2	L-Phenylalanine‡	724	14.5	50,000	756	66
4	Cinnamic acid§	568	10.9	52,200	350	148
4	Cinnamic acid-α-C^{14}	1,360	15.1	90,100	563	160
4	Cinnamic acid-β-C^{14}	1,295	13.8	93,800	543	173
2	Cinnamic acid (ring and β-labelled)	1,600	17.4	92,000	271	338
2	*p*-Hydroxycinnamic acid-β-C^{14}	457	14.5	31,500	170	185
2	Caffeic acid-β-C^{14}	527	13.3	39,600	31	1,268
3	Sinapic acid-β-C^{14}	795	12.5	63,600	15	4,240
4	*m*-Methoxycinnamic acid-β-C^{14}	1,000	9.6	104,100	23	4,502
5	Phenylacetic acid§	830	16.6	50,100	7	7,260
3	Protocatechuic acid§	1,385	17.2	80,500	7	11,840
5	*p*-Hydroxybenzoic acid§	1,260	12.2	103,200	7	14,350
3	Ferulic acid-β-C^{14}	1,546	17.8	86,800	0.15	578,000

*Specific activity of compound administered divided by specific activity of quercetin.
†Calculated from analysis of the recrystallized pentaacetate.
‡Randomly labelled.
§Carboxyl labelled.

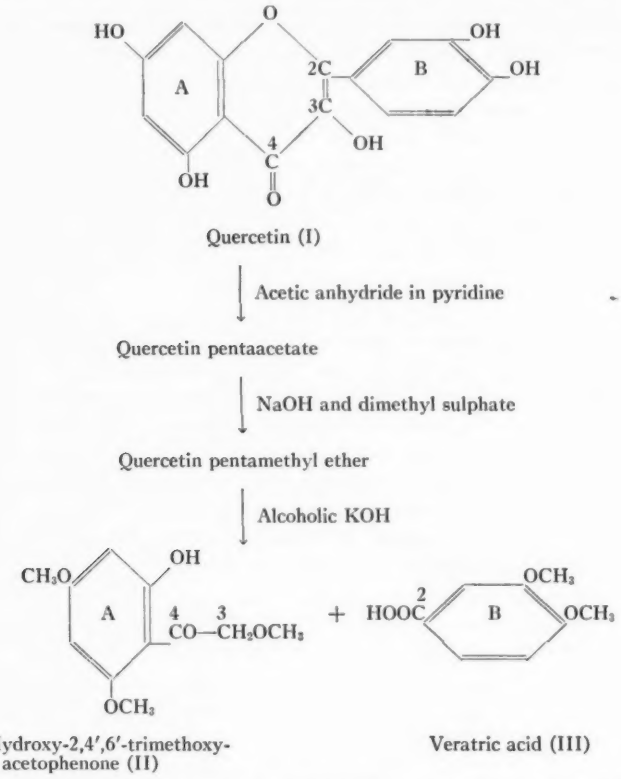


FIG. 1. Degradation of quercetin. The numbers on the formulae II and III are to indicate which carbon atoms of quercetin the numbered carbons represent.

TABLE II

DISTRIBUTION OF C^{14} IN QUERCETIN

Precursor administered to plants	% of total activity of quercetin* found in:	
	2′-Hydroxy-2,4′,6′-trimethoxyacetophenone (II)	Veratric acid (III)
L-Phenylalanine (randomly labelled)	22.8	78.4
Cinnamic acid (carboxyl labelled)	94.3	1.7
Cinnamic acid-α-C^{14}	102	0.2
Cinnamic acid-β-C^{14}	0.2	98.0
Cinnamic acid (ring and β-labelled)	0.2	99.0
p-Hydroxycinnamic acid-β-C^{14}	0.7	99.5
Caffeic acid-β-C^{14}	9.0	85.5

*Calculated from analysis of quercetin pentamethyl ether.

In addition to the compounds listed in Table I a number of amino acids were tested as precursors of quercetin in the same way. The amino acids used were the randomly-labelled L-isomers of proline, arginine, glycine, threonine, leucine, isoleucine, alanine, aspartic acid, glutamic acid, lysine, valine, and tyrosine. Of these, glutamic acid was found to be the best precursor of quercetin but it was only one-seventh as effective as phenylalanine administered under the same conditions. The other amino acids were even less effective than this and tyrosine, in spite of its close structural relationship to phenylalanine, was only one twenty-eighth as effective as a precursor of quercetin.

Degradation of quercetin, as outlined in Fig. 1, has given results (Table II) which indicate that the $C_6.C_3$ skeletons of such precursors as phenylalanine and the cinnamic acid derivatives enter into the flavonol molecule as a unit, giving rise to ring B and the adjacent carbons 2, 3, and 4 of the heterocyclic ring. On the other hand there is no reason to believe that they contribute appreciably to formation of ring A. For example cinnamic acid labelled in the ring or β-carbon was converted by the plant into quercetin with practically all of the activity in the part of the molecule obtained as veratric acid. This was also true with β-labelled *p*-hydroxycinnamic acid, and, to a lesser extent, for caffeic acid-β-C^{14}. Cinnamic acid-α-C^{14} would be expected to give quercetin labelled in carbon 3 while carboxyl-labelled cinnamic acid should give quercetin labelled in carbon 4. The acetophenone derivative (III), obtained from the quercetin samples formed from these precursors, contained nearly all of the activity, as expected. The quercetin formed from randomly-labelled L-phenylalanine would be expected to have two-ninths (22.3%) of the C^{14} in carbons 3 and 4 combined, with the remaining 77.7% in ring B and carbon 2. The results obtained (Table II) agree closely with these predicted values.

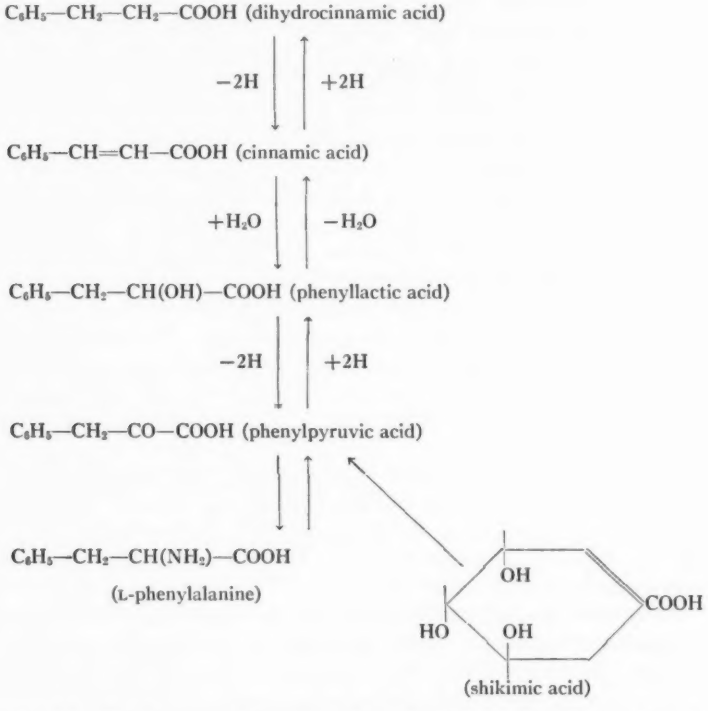
The quercetin formed from shikimic acid was degraded by a different procedure (21) which gave phloroglucinol (ring A) and protocatechuic acid (ring B and carbon 2) as the products. The protocatechuic acid contained 93% of the total C^{14} activity in the quercetin molecule while phloroglucinol accounted for only 3.6%.

Some idea of the reproducibility of the experiments can be obtained by comparing the dilution values obtained for the differently-labelled forms of cinnamic acid (Table I). Since the results obtained by degrading quercetin (Table II) indicated that the cinnamic acid carbon skeleton entered the flavonol molecule as a unit, the dilution values should be the same. The agreement is close for plants of the same batch (Table I) but there was a variation in dilution values (about 2:1) when plants from different batches were compared.

Discussion

It appears, from the foregoing results, that the ring B and carbons 2, 3, and 4 of quercetin are formed in buckwheat from an aromatic compound with a $C_6.C_3$ skeleton. A number of precursors with this skeleton can be

converted to quercetin. A similar situation was found to exist in the biosynthesis of lignin in wheat and Manitoba maple (2, 3, 4) and, indeed, all the compounds found to be good precursors of quercetin are also good precursors of lignin. In addition compounds such as ferulic and sinapic acid are good precursors of lignin, as might be expected, since they contain the guaiacyl and syringyl nuclei, respectively. The practically universal occurrence of lignin and the widespread distribution of flavonoids in higher plants suggest that an active metabolic pool for the interconversion of $C_6.C_3$ compounds is a characteristic feature of plant metabolism. It is possible to deduce some of the enzymatic reactions which may occur in this pool from the results in this and previous papers (2, 3, 4).



The comparable effectiveness of compounds such as shikimic acid, L-phenylalanine, and cinnamic acid as precursors of quercetin and lignin can be readily understood if they are interconvertible as shown above. In this scheme phenylpyruvic acid is a central intermediate. Davis (7) and co-workers have shown that phenylpyruvic acid is the first aromatic $C_6.C_3$ compound formed in *Escherichia coli*, and that shikimic acid is an important intermediate in its formation. The formation of phenylpyruvic acid from phenylalanine results from a well-known enzymatic transamination. In wheat, dihydrocin-

namic acid is as good as cinnamic acid as a precursor of lignin. The reactions shown for interconversion of these compounds to phenylpyruvic acid are analogous to those responsible for the interconversion of succinic, fumaric, malic, and oxaloacetic acids in the well-known Krebs citric acid cycle.

The reactions shown above are only a few of those which may occur in the $C_6.C_3$ metabolic pool. The ready conversion of *p*-hydroxycinnamic acid to both quercetin and lignin and the formation of lignin from caffeic, ferulic, and sinapic acids suggest that these same changes may occur with units containing *p*-hydroxyphenyl, 3,4-dihydroxyphenyl, guaiacyl, and syringyl groups as well as with those containing unsubstituted phenyl groups. Hydroxylations of the aromatic nucleus, such as are catalyzed by phenolase, must occur and may not be readily reversible reactions. Other possible reactions in the $C_6.C_3$ metabolic pool include methylation of phenolic hydroxyl groups and reduction of the carboxyl groups, e.g. reduction of ferulic acid to coniferyl alcohol. These reactions would be expected to yield the monomeric intermediates necessary for lignification. Hibbert (13) has also postulated a number of enzymatic reactions in plants involving $C_6.C_3$ metabolites with a guaiacyl or a syringyl nucleus and a terminal methyl group in the side chain. Higher plants probably contain many enzymes involved in the turnover of $C_6.C_3$ metabolites and should provide a fertile field for enzymological investigations.

The ineffectiveness of tyrosine as a precursor of quercetin is in agreement with previous work showing its low efficiency as a precursor of lignin in buckwheat (4). Since *p*-hydroxycinnamic acid is a good precursor of both quercetin and lignin it seems probable that tyrosine is deaminated with difficulty in buckwheat plants. Previous work on lignin formation (4) has shown that tyrosine is a good precursor in some species but not in others.

The results obtained in this study of quercetin synthesis in buckwheat agree in a general way with the scheme of Birch, Donovan, and Moewus (1) for biosynthesis of quercetin in *Chlamydomonas eugametos*. It appears from both lines of investigation that quercetin arises by reactions involving condensation of a $C_6.C_3$ aromatic precursor with some other reactant to give the final $C_6.C_3.C_6$ structure. However the $C_6.C_3$ compounds appear to differ in the two organisms. Caffeic acid and L-tyrosine were found to be good precursors of quercetin in *Chlamydomonas* while *p*-hydroxycinnamic acid and cinnamic acid were ineffective. The reverse was found to be true for buckwheat. Both organisms can utilize L-phenylalanine, but not ferulic acid, for quercetin biosynthesis. It appears that the $C_6.C_3$ compound may be fully hydroxylated before condensation in the alga, while in buckwheat, condensation may occur before the second hydroxyl is introduced into ring B.

Acknowledgments

The authors are indebted to Mr. M. D. Chisholm and Mr. J. Dyck for valuable technical assistance and to Dean W. C. MacAulay, College of Pharmacy, University of Saskatchewan, for permission to publish data from a thesis (20).

References

1. BIRCH, A. J., DONOVAN, F. W., and MOEWUS, F. Nature, **172**, 902 (1953).
2. BROWN, S. A. and NEISH, A. C. Nature, **175**, 688 (1955).
3. BROWN, S. A. and NEISH, A. C. Can. J. Biochem. Physiol. **33**, 948 (1955).
4. BROWN, S. A. and NEISH, A. C. Can. J. Biochem. Physiol. **34**, 769 (1956).
5. BUCHANAN, D. L. and NAKAO, A. J. Am. Chem. Soc. **74**, 2389 (1952).
6. COUCH, J. F., NAGHSKI, J., and KREWSON, C. F. Science, **103**, 197 (1946).
7. DAVIS, B. D. Advances in enzymology. Interscience Publishers Inc., New York. Vol. 16. 1955. p. 247.
8. ELLIS, C., SWANEY, M. W., and EASTWOOD, T. Soilless growth of plants. 2nd ed. Reinhold Publishing Corporation, New York. 1947.
9. FREUDENBERG, K. Ann. **433**, 263 (1923).
10. GEISHMANN, T. A. and HINREINER, E. Botan. Rev. **18**, 77 (1952).
11. GRIFFITH, J. Q., JR., KREWSON, C. F., and NAGHSKI, J. Rutin and related flavonoids. Mack Pub. Co., Easton, Pa. 1954.
12. HERZIG, J. and HOFMANN, B. Chem. Ber. **42**, 157 (1909).
13. HIBBERT, H. Ann. Rev. Biochem. **11**, 183 (1942).
14. JACOBSON, L. Plant Physiol. **26**, 411 (1951).
15. MCGREGOR, W. G. and MCKILLICAN, M. W. Sci. Agr. **32**, 48 (1952).
16. PAECH, K. Ann. Review Plant Physiol. **6**, 281 (1955).
17. PERKIN, A. G. J. Chem. Soc. **99**, 1721 (1911).
18. RAO, P. S. and SESHADRI, T. R. Proc. Indian. Acad. Sci. **9A**, 177 (1939).
19. SESHADRI, T. R. Ann. Rev. Biochem. **20**, 487 (1951).
20. UNDERHILL, E. W. The biosynthesis of quercetin in buckwheat. M.Sc. Thesis, College of Pharmacy, University of Saskatchewan. 1956.
21. WATKIN, J. E., UNDERHILL, E. W., and NEISH, A. C. Can. J. Biochem. Physiol. **35**, 229 (1957). (Part II of this series.)

BIOSYNTHESIS OF QUERCETIN IN BUCKWHEAT

PART II[1]

J. E. WATKIN,[2] E. W. UNDERHILL,[3] and A. C. NEISH

Abstract

Various C^{14}-labelled compounds were administered to *Fagopyrum tataricum*. After allowing 24 hours for metabolism the quercetin was isolated and degraded, by a modified alkaline fusion, into phloroglucinol (ring A) and protocatechuic acid (ring B plus carbon 2). Uniformly labelled sucrose or $C^{14}O_2$ (assimilated photosynthetically) formed quercetin equally labelled in the A and B rings, but sodium acetate-1-C^{14} (or acetate-2-C^{14}) gave quercetin labelled almost entirely in ring A. Isotope competition experiments indicated *meso*-inositol and phloroglucinol were not precursors of ring A. The biosynthesis of quercetin from $C^{14}O_2$ or acetate required the combined action of stem and leaves. Light was not required for synthesis of quercetin from acetate.

Introduction

Ring A of quercetin and other flavonoids is believed to be derived from phloroglucinol. Robinson (12) suggested 20 years ago that this ring, in anthocyanidins, was formed from phloroglucinol. It has been proposed that phloroglucinol is formed from *meso*-inositol. Kursanov (8) has shown that the polyphenols with the phloroglucinol structure in tea leaves increase in amount when the leaves are infiltrated with *meso*-inositol. Birch, Donovan, and Moewus (2) found that *meso*-inositol and phloroglucinol were both able to act as precursors of quercetin in *Chlamydomonas*. However Birch and Donovan (1) have suggested another route for the biosynthesis of phloroglucinol derivatives which involves acetate rather than inositol and does not require the participation of phloroglucinol itself. The present paper is concerned with the origin of ring A of quercetin in buckwheat. The results support the acetate theory (1).

Quercetin can be split by fusion in potassium hydroxide into phloroglucinol (from ring A) and protocatechuic acid (from ring B plus carbon 2) with the loss of carbons 3 and 4. Yields are usually poor. In this work a method of alkaline digestion has been developed which gives excellent yields. The degradation products can be sufficiently purified as their acetates to allow the calculation, by difference, of the average activity of the lost carbon atoms with a fair degree of accuracy.

The formation of quercetin was studied by giving buckwheat cuttings simple labelled compounds as in Part I (15). When labelled precursors were not available the unlabelled compounds were tested by use of an isotope competition technique (11). When $C^{14}O_2$ is given to buckwheat the quercetin

[1]Manuscript received October 9, 1956.
Contribution from the Prairie Regional Laboratory, National Research Council, Saskatoon, Sask.
Issued as N.R.C. No. 4211.
[2]Postdoctorate Fellow, National Research Council, 1955–1956.
[3]Graduate Student assisted by National Research Council Studentship, College of Pharmacy, University of Saskatchewan, Saskatoon, Sask.

formed should be evenly labelled as it must all be derived ultimately from products of photosynthesis. If an unlabelled compound that is a precursor to ring A (but not ring B) is given at the same time as the $C^{14}O_2$, the pool of that precursor should be diluted and its specific activity correspondingly reduced. Thus the specific activity of ring A would be reduced in comparison with ring B. The total synthesis of quercetin should not be affected by the use of excess precursor to one ring as the precursors to the other ring would become limiting. The distribution of activity in the molecule should be unaffected by the total amount of quercetin made or the effectiveness of the substance used as a precursor. A limitation of the method is that the amount of substance introduced has to be several times the natural concentration, which is often unknown, and sometimes toxic effects ensue.

Experimental

Materials

The C^{14}-labelled compounds were purchased from Atomic Energy of Canada. The buckwheat plants were the same variety and were grown in the same manner as described in Part I (15).

Administration of Precursors

Solutions of precursors were administered to buckwheat tops in the manner previously described (15). Experiments with $C^{14}O_2$ were carried out in a large glass jar, 24 in. high and 12 in. in diameter, which was sunk by lead disks in an aquarium, 20 in. square by 22 in. high, full of flowing water. The jar was covered with a plate glass lid ground to an airtight fit and having four 1 in. holes. These holes were closed with rubber stoppers carrying through them (*a*) a cable from a Tracerlab SU-3B laboratory monitor to its counter tube suspended in the jar, (*b*) a cable to a fractional H.P. induction motor driving a 2 in. air fan, (*c*) and (*d*) $\frac{1}{4}$ in. glass tubes for air entry and exit. The entry tube was connected to a small bubbler with a side arm closed by a rubber serum bottle cap. The exit tube was joined to a water manometer and was connected to a water pump. Photoflood bulbs (300 w.) shone through the aquarium onto the shoot in the jar giving a light intensity of 1500–2000 ft-c. in the jar. The water served the double purpose of removing most of the intense infrared heat whilst permitting almost all the light to pass, and of dissipating the small amount of heat developed in the jar. With the fan circulating the air over the walls of the jar, the internal air temperature was only 3° C. higher than the external water temperature so that the relative humidity in the jar did not fall below 83%. This was important, as the buckwheat grown indoors during the winter had a low dry matter content and the cuttings wilted easily if the humidity was low.

The jar, with the cuttings inside, was evacuated to a negative pressure of 2 ft. of water. C^{14}-Labelled sodium carbonate in dilute alkali was put in the bubbler and dilute acid injected through the side arm using a hypodermic syringe. As the carbon dioxide was released a current of air was allowed to bubble through into the jar sweeping the carbon dioxide with it until a

slight negative pressure remained. Uptake of the carbon dioxide by photosynthesis in the shoot was followed by reduction of the counter reading. When most of the $C^{14}O_2$ was assimilated the remainder was swept from the jar by a current of air and absorbed in alkali.

Isolation of Quercetin

Fresh plant material (50 g.) was cut into large pieces in a beaker and boiling absolute ethanol (200 ml.) poured over it. After 5 minutes' boiling the plant material was macerated in a Waring blendor for 2 minutes and the extraction continued for 5 minutes more. The marc was separated by filtration and extracted again with 200 ml. of boiling 80% ethanol. Finally the marc was washed on the filter with 100 ml. of boiling 80% ethanol. The combined filtrates were worked up as described in Part I as far as the isolation of purified quercetin pentaacetate (15).

Degradation of Quercetin

Quercetin pentaacetate is not wetted by aqueous potassium hydroxide so a preliminary partial hydrolysis was carried out by ammonium hydroxide in acetone. Quercetin pentaacetate (80–90 mg.) was dissolved in 5 ml. of warm (50° C.) acetone in a 20 ml. platinum crucible and a few drops of ammonia added. After 15 minutes the acetone was evaporated by a jet of cold air and the residue dissolved in 10 ml. of 50% (w/w) aqueous potassium hydroxide. A knife point of sodium formate was added and the crucible was nearly filled with xylene. It was then heated on a steam bath for 5 hours. The production of phloroglucinol was followed by removing 0.01 ml. of the alkaline layer, pipetting it into 10 ml. of Lindz reagent* (9), and after 5 minutes measuring the orange color formed, by a colorimeter set at 500 mμ. Degradation of the quercetin proceeded at a constant rate of about 10 mg./hour until complete and was independent of the concentration of quercetin. A platinum crucible was essential, since reaction was very slow in glass. When the theoretical yield of phloroglucinol was reached the crucible was cooled and the contents were washed out with 30 ml. of water. After the xylene layer had been separated, the aqueous layer was acidified with 10 ml. of hydrochloric acid and passed through a 20 mm. by 30 mm. column of equal weights of celite 535 and Darco-G-60 charcoal. The column was washed with water until the washings were neutral. The phloroglucinol was eluted first, using 80 ml. of 50% ethanol, and then the protocatechuic acid, using 50 ml. of 20% benzene in ethanol. The colorless solutions were evaporated under an air jet and the residues separately taken up in 1 ml. of equal volumes of acetic anhydride and pyridine. After 1 hour the solvents were evaporated to give crude residues of phloroglucinol triacetate and protocatechuic acid diacetate.

The phloroglucinol triacetate was dissolved in 5 ml. dry acetone, adsorbed on a small magnesol† column, and eluted with 30 ml. of a mixture of equal volumes of ethanol and acetone. After the eluate had been evaporated

*162 mg. of vanillin in 16 ml. of alcohol, made to 100 ml. with hydrochloric acid.

†Magnesol is a synthetic hydrated magnesium silicate. It was obtained from the Westvaco Chlorine Products Co., South Charleston, West Virginia.

to 2–3 ml., 30 ml. of hot petrol (b.p. 80°–100° C.) was added and, when the ethanol was evaporated under an air jet, the product crystallized in very fine needles, m.p. 104° C.

The protocatechuic acid diacetate was extracted from the residue with a few milliliters of hot benzene and recrystallized twice from 2 ml. of hot water, m.p. 158° C.

The activities of the compounds were measured in a gas-phase counter after combustion to carbon dioxide (5).

Results

The dilution values in Table I are an indication of the relative efficiency of sugars, acetate, and glycerol as precursors of quercetin in buckwheat tops. The actual dilution will depend on the size of the metabolic pool that the precursor enters, as well as on the amount of precursor converted to quercetin and the amount of inactive quercetin present in the plant.

In order to obtain a measurement indicative of the amount of precursor converted to quercetin this experiment was carried out with plants grown in the same batch and the precursors were administered in approximately the same doses. The dilution value appears to be inversely proportional to the dose over a small range. Thus for the acetates (Table I) the ratio of dose was 1.62 and the inverse ratio of dilutions was 1.57. The ratio of doses of sucrose and glucose was 1.65 and the inverse ratio of dilutions 1.72.

It appears (Table I) that quercetin is formed more readily from acetate than from sugars. However the smaller dilution of acetate compared to sugars may be because acetate enters a smaller pool and is not diluted as much as the sugars are by photosynthesis during the 24 hour period allowed for metabolism. Quercetin formed from uniformly-labelled sucrose is labelled about equally in both rings but quercetin formed from acetate is labelled predominantly in ring A (Table II, lines 5, 6, 7). These data are consistent with the hypothesis that ring A of quercetin is formed from acetate groups, which arise naturally from carbohydrate.

TABLE I

RELATIVE EFFICIENCY OF COMPOUNDS AS QUERCETIN PRECURSORS

Compound administered	Specific activity, mμc./mM. of C	Dose as μM. of C per g. dry wt.	Activity quercetin isolated, mμc./mM. of C	Dilution value*
1. Glycerol-1,3-C^{14}	345,000	178	171	2,020
2. Glucose†	45,800	109	35.7	1,285
3. Sucrose†	45,500	180	60.8	748
4. Sodium acetate-1-C^{14}	69,300	109	94.8	732
5. Sodium acetate-2-C^{14}	87,300	67	76.2	1,145

*Specific activity of compound administered (column 2) divided by specific activity of quercetin (column 4).

†Uniformly labelled with C^{14}.

TABLE II

DISTRIBUTION OF ACTIVITY IN THE QUERCETIN MOLECULE

Dose given per g. dry wt.	Activity quercetin isolated, mμc./mM. of C	Relative specific activity of carbon; quercetin = 100		
		Ring A	Ring B + C_3	$C_3 + C_4$
1. $C^{14}O_2$ only, 635 μM. 24.5 μc.	610	91	95	142
2. $C^{14}O_2$ plus 29 μM. inactive phenylalanine	56	248	22	0
3. $C^{14}O_2$ plus 64 μM. inactive *meso*-inositol	517	98	97	118
4. $C^{14}O_2$ plus 99 μM. inactive phloroglucinol	70	93	106	102
5. 33.5 μM. methyl-labelled acetate	76.2	247	1.6	3.6
6. 54.6 μM. carboxyl-labelled acetate	94.8	242	4	8.9
7. 15 μM. uniformly-labelled sucrose	60.8	103	92	124

An attempt was made to identify other possible precursors of ring A by isotope dilution experiments (Table II, lines 1, 2, 3, 4). In order to test the technique an experiment was done with L-phenylalanine, which has been established as a good precursor of ring B by experiments using phenylalanine-C^{14} (15). When buckwheat cuttings were given $C^{14}O_2$, only, the quercetin synthesized was equally labelled in the A and B rings. When unlabelled phenylalanine and $C^{14}O_2$ were administered simultaneously the activity of ring B was only one-tenth that of ring A. This indicates that phenylalanine diluted the natural pool of ring B precursors as expected. When unlabelled *meso*-inositol or phloroglucinol were administered simultaneously with $C^{14}O_2$, the quercetin formed was uniformly labelled. Bonner (3) gives 0.1% to 1% as the concentration of *meso*-inositol in green plants. The amount administered in the above experiment (1.15% of the dry weight) should have caused a detectable dilution. The phloroglucinol administered (1.25% of the dry weight) caused wilting and a decrease in the over-all amount of quercetin synthesized. It was concluded that inositol and phloroglucinol are not quercetin precursors in buckwheat, as has been reported for *Chlamydomonas* (2).

A single detached leaf of buckwheat, given $C^{14}O_2$, assimilated it readily (by photosynthesis) but the quercetin formed had only 1/60 of the activity expected by comparison with whole shoots. A stem devoid of leaves, although green, took up hardly any carbon dioxide. Quercetin was isolated separately from the stem and leaves of a shoot after 5 hours' photosynthesis in $C^{14}O_2$ (Table III). The specific activity of the stem and leaf tissue was equal but the specific activity of the quercetin from the stem was four times that of the quercetin from the leaves of the same shoot. This suggests that quercetin may be synthesized in the stem and stored in the leaves.

Table IV gives the results of an experiment designed to show whether quercetin could be made from acetate in a stem devoid of leaves. Three cuttings from the same batch of plants were given sodium acetate-1-C^{14}

TABLE III

DISTRIBUTION OF ACTIVITY OF QUERCETIN IN LEAVES AND STEMS

	Leaves	Stems
Specific activity of $C^{14}O_2$, mμc./mM.	20,000	20,000
Total activity found, μc./g.	12.2	12.2
Rutin isolated (% of dry weight)	3	0.85
Specific activity of quercetin, mμc./mM. of C	116	440
Dilution factor*	172	45.5

*Specific activity of CO_2 divided by specific activity of quercetin.

TABLE IV

SYNTHESIS OF QUERCETIN BY STEMS

	After 1½ hours	After 24 hours	
	Stem not defoliated (Cutting 1)	Stem defoliated after 1½ hr. (Cutting 2)	Stem not defoliated (Cutting 3)
Sodium acetate-1-C^{14} administered (mg.)	6.9	5.8	7.5
Sodium acetate-1-C^{14} administered (μc.)	10.2	8.6	11.1
Total activity found in leaves (μc.)	3.6	2.5	—
Total activity found in stems (μc.)*	6.6	6.1	—
Specific activity of quercetin (mμc./mM. of C)‡	29.7	46.0	90.0
Total amounts of C^{14} found in quercetin (mμc.)‡	5.7	11.0	74.0
Rate of C^{14} incorporation into quercetin (mμc. per hour)†	3.8	0.23	3.1
Rutin isolated as % of dry weight	0.5	0.5	2.0

*By difference.
†Total amount of C^{14} found in quercetin (mμc.) divided by duration of experiment in hours.
‡The quercetin was obtained from the stems only in Cuttings 1 and 2 and from the whole cutting in Expt. 3.

through the cut ends in the usual way. It was necessary to use stems carrying leaves for the operation since absorption of the acetate solution depends on transpiration. After 1½ hours, when absorption of the solution was complete, the leaves were removed from two of the cuttings by severing the petiole near the leaf blade. At this time most of the C^{14} was still in the stems. One of the stems was extracted at once (Cutting 1). The other stem (Cutting 2) was extracted after being allowed to metabolize further, for 24 hours in all. At the end of this time it was still in good condition and had not lost weight. The difference in the amounts of quercetin formed from acetate in these stems represents the quercetin formed during 22½ hours' metabolism without leaves. The other shoot (Cutting 3) was allowed to metabolize 24 hours with its leaves in place. The amount of quercetin formed in Cutting 3 should be roughly equivalent to that which would have been formed in Cutting 2 if quercetin synthesis was not affected by defoliation. The whole cutting

was worked up to obtain any quercetin formed in the stems and translocated to the leaves. The average rate of quercetin synthesis in Cutting 3 during 24 hours was about the same as in the stem of Cutting 1 during 1½ hours, when translocation of quercetin would be relatively unimportant. It is evident that quercetin was synthesized in the stem at a rate comparable to that of the whole cutting and that removal of the leaves causes a great decrease in this rate. Since detached leaves cannot synthesize quercetin, it appears the quercetin formation is a process requiring the combined action of stems and leaves.

Light is required for synthesis of anthocyanins in algae (14) so it is possible that it may be essential for quercetin formation also. An experiment (Table V) with buckwheat cuttings showed that light was not essential for quercetin synthesis from acetate.

TABLE V

EFFECT OF LIGHT ON QUERCETIN SYNTHESIS IN BUCKWHEAT CUTTINGS

	Dark	Light
1. Dose of acetate (μM./g.)	35	33.5
2. Specific activity of acetate (μc./mM. of C)	69.3	69.3
3. Specific activity of quercetin isolated (mμc./mM. of C)	167	78
4. Dilution factor*	415	890

*Line 2 converted to mμc. and divided by line 3.

Discussion

The previous paper (15) has shown that in biosynthesis of quercetin by buckwheat plants ring B and carbons 2, 3, and 4 can be formed from a $C_6.C_3$ compound which is incorporated as a unit. The results in this paper show that ring A can be readily formed from acetate. The data do not support the hypothesis that phloroglucinol and *meso*-inositol are precursors of ring A as has been suggested (2) and, indeed, it is difficult to envisage any close metabolic relation between inositol and acetate.

Birch and Donovan (1) have proposed an ingenious theory for the origin of phloroglucinol derivatives from acetate units. They noted that the structure of a large number of naturally occurring orcinol and phloroglucinol derivatives could be explained by assuming they originated by cyclization polyketo acids formed by head to tail condensation of acetate units. For example one might suppose that three acetate units could condense head to tail forming 3,5-dioxo-*n*-hexanoic acid. If the head of a molecule of this acid condensed with its own tail then phloroglucinol would be formed. Free phloroglucinol would not necessarily be an intermediate in the synthesis of flavonoids. If the three acetate units condensed on the carboxyl carbon of a $C_6.C_3$ acid, then cyclization would give a $C_6.C_3.C_6$ compound with the phloroglucinol pattern of hydroxylation in the newly-formed ring.

If this theory is used to explain the biosynthesis of quercetin in buckwheat (see Fig. 1) a scheme is obtained which is in agreement with all the observations made so far. Phenylpyruvic acid was chosen as the $C_6.C_3$ acid because it has the right level of oxidation to give a flavonol. If three acetate units condense on it in the manner postulated (1), then 3,5,7,8-tetraoxo-9-phenyl-*n*-nonoic acid would be formed. This is shown in Fig. 1, as the fully-enolized form (I). Condensation of the carboxyl carbon of I with carbon 6 of the same molecule would give the chalcone derivative II. Cyclization of II by oxidation would give a flavonol.

In order to obtain quercetin it is necessary to put hydroxyls on ring B at some stage. The hydroxylation pattern of this ring is what would be expected if the hydroxyls were introduced by phenolase action (10). Since *p*-hydroxycinnamic acid is a good precursor of quercetin (15) the *para* hydroxyl might be

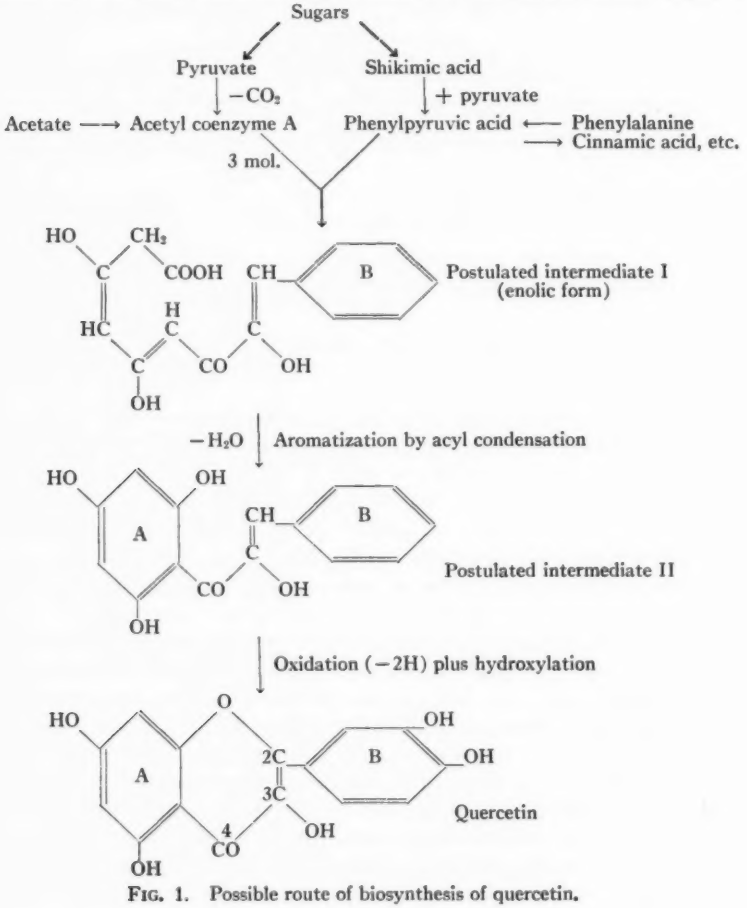


FIG. 1. Possible route of biosynthesis of quercetin.

introduced at the phenylpropanoid stage. *p*-Hydroxyphenylpyruvic acid would then be substituted for phenylpyruvic acid in Fig. 1. However 3,4-dihydroxycinnamic acid (caffeic acid) is not a good precursor of quercetin (15) so it is probable that the other hydroxyl of quercetin is introduced at a late stage in the synthesis. There are not sufficient data to decide when hydroxylation of the B ring might occur but it is tempting to postulate (as in Fig. 1) that it is the final step in biosynthesis of quercetin since this allows I and II to be considered as common intermediates in the biosynthesis of a variety of flavonols.

For the postulated reactions (Fig. 1) to proceed readily, active acetate units (acetyl coenzyme A) and similar activated forms of the $C_6.C_3$ acid and I would be required. The final closing of the heterocyclic ring by oxidation of II has a chemical analogy in the oxidation of 2-hydroxychalcones to flavonols by dilute hydrogen peroxide (7). A similar reaction (oxidation of a hydroxychalcone to a benzalcoumarone) is catalyzed by an enzyme found in *Coreopsis lanceolata* (13).

It appears that higher plants have at least two basically different pathways for synthesizing benzenoid compounds. The first pathway, which involves shikimic acid, leads to phenylpropane derivates. This was first established in bacteria by the outstanding researches of Davis and his collaborators (6). It seems likely that precursors of lignin (4) and ring B of quercetin (15) are formed by this route in higher plants. The second pathway involves condensation of acetate units, as suggested by Birch and Donovan (1), and gives rise to phloroglucinol derivatives. This route appears to function in formation of ring A of quercetin in buckwheat. The ability to synthesize a wide variety of benzenoid compounds is a characteristic feature of plant metabolism and, judging from their structure, it is probable that other aromatic compounds (e.g. thymol) are formed by pathways quite different from the two indicated above.

Acknowledgment

The authors are indebted to Mr. M. D. Chisholm and Mr. J. Dyck for valuable technical assistance.

References

1. Birch, A. J. and Donovan, F. W. Australian J. Chem. **6**, 360 (1953).
2. Birch, A. J., Donovan, F. W., and Moewus, F. Nature, **172**, 902 (1953).
3. Bonner, J. Plant biochemistry. Academic Press, Inc., New York. 1950. p. 43.
4. Brown, S. A. and Neish, A. C. Nature, **175**, 688 (1955).
5. Buchanan, D. L. and Nakao, A. J. Am. Chem. Soc. **74**, 2389 (1952).
6. Davis, B. D. Advances in Enzymol. **16**, 247 (1955).
7. Geissman, T. A. and Fukushima, D. K. J. Am. Chem. Soc. **70**, 1686 (1948).
8. Kursanov, A. L. Chem. Abstr. **46**, 2710*h* (1952).
9. Lindt, O. Z. anal. Chem. **26**, 260 (1887).
10. Mason, H. S. Advances in Enzymol. **16**, 105 (1955).
11. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J. Carnegie Inst. Wash. Publ. **607**, 406 (1955).
12. Robinson, R. Nature, **137**, 172 (1936).
13. Shimokoriyama, M. and Hattori, S. J. Am. Chem. Soc. **75**, 2277 (1953).
14. Thimann, K. Y. and Radner, B. S. Arch. Biochem. Biophys. **59**, 511 (1955).
15. Underhill, E. W., Watkin, J. E., and Neish, A. C. Can. J. Biochem. Physiol. **35**, 219 (1957).

NOTES

A NOTE ON THE INFRARED SPECTRA OF α-CEPHALINS

ERICH BAER[1]

The increasing use of spectroscopic methods for the identification of naturally occurring compounds prompted us several years ago to record the infrared spectra of our synthetic dimyristoyl (DMC), dipalmitoyl (DPC), and distearoyl (DSC) L-α-cephalins (1). As was to be expected, the infrared spectra of the homologous compounds were quite similar. However, some slight differences in the region of 11.5–14μ were noted which possibly were caused by impurities. Efforts to remove these have been rewarded finally by products with more uniform spectra and possessing melting points that are higher than those previously reported by us (1). The improvements in the purification procedure that are given below should be applied to products prepared by our previously described method.

Experimental Part

Dimyristoyl, dipalmitoyl, and distearoyl L-α-cephalin were prepared by the procedure of Baer, Maurukas, and Russell (1) and treated as follows: One gram of the cephalin was suspended with stirring for 10 minutes in 15 ml. of dilute acetic acid (25%), and the mixture was separated by centrifugation. The precipitate was treated in the same manner successively with two 15 ml. portions of fresh acetic acid and three 10 ml. portions of anhydrous acetone. The acetone was removed in an air current and the cephalin was dried *in vacuo* over solid sodium hydroxide. For recrystallization, the cephalin was dissolved in chloroform (23 ml., 35 ml., or 85 ml. of solvent per 1 g. of DMC, DPC, or DSC, respectively) and after twice the volume of methanol was added, the solution was set aside at room temperature (20°–25°) until spontaneous crystallization set in. The mixture was then placed in an icebox at 5° for 12 hours. The cephalins, when permitted to crystallize undisturbed, formed coarse granular precipitates (spherulites). The precipitates were collected with suction on a Büchner funnel, washed with pure ether, and dried *in vacuo*. The cephalins were recovered in yields ranging from 72% for DSC to 82% for DMC. They began to sinter slightly at about 130°–135°, turned amber at about 170°, and coalesced suddenly with the formation of a meniscus[2] at 195°–196° (DMC), 186°–187° (DPC), and 180°–182° (DSC). Their specific rotations in a mixture of dry and ethanol-free chloroform and glacial acetic acid (9:1, v/v) were: DMC, +6.7° (*c*, 8.5); DPC, +6.4° (*c*, 7.1); DSC, +6.0° (*c*, 4.5).

[1]This work was supported by a Summer Research Associateship for 1956 from the National Research Council, Ottawa.

[2]The melting point determinations were carried out in capillary tubes using an electrically heated bath of *n*-butylphthalate and short-stem thermometers. The temperature of the bath was raised at a rate of 15°–20° per minute up to 150°, and 4°–5° thereafter.

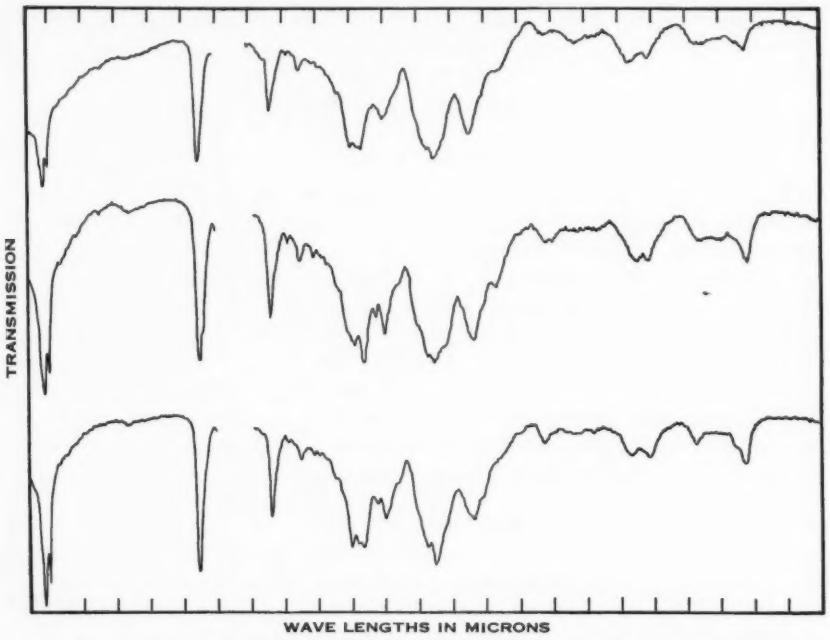


FIG. 1. Infrared spectra of dimyristoyl (upper curve), dipalmitoyl (middle curve), and distearoyl L-α-cephalin (lower curve). A Perkin–Elmer (Model 21) double-beam spectrophotometer (sodium chloride prism) was used. The cephalin sample was ground mechanically in a tool steel mortar with potassium bromide for 15 minutes at room temperature. The die (PE-2) was evacuated to 0.06 mm. Hg and the disk was pressed at 23,000 lb. for 10 minutes. Sample concentration: 0.5 mg. of DMC and DPC, and 1.0 mg. of DSC per 1.0 g. of KBr.

Acknowledgment

The infrared spectra were obtained through the courtesy of Dr. G. V. Marinetti of the Department of Biochemistry and of Dr. W. B. Mason of the Atomic Energy Project of the University of Rochester School of Medicine and Dentistry, and were made possible in part by funds from the United States Atomic Energy Commission. The preparation of the cephalins by Mr. Hans Flehmig is gratefully acknowledged.

References

1. BAER, E., MAURUKAS, J., and RUSSELL, M. J. Am. Chem. Soc. **74**, 152 (1952).

RECEIVED NOVEMBER 6, 1956.
BANTING AND BEST DEPARTMENT OF MEDICAL RESEARCH,
UNIVERSITY OF TORONTO,
TORONTO, CANADA.

COMMONWEALTH BUREAU OF ANIMAL NUTRITION

ROWETT RESEARCH INSTITUTE, BUCKSBURN, ABERDEENSHIRE, SCOTLAND

Publications and Services

NUTRITION ABSTRACTS AND REVIEWS

World literature on the nutrition of man and animals is abstracted in sections embracing methods of analysis and experimental techniques; composition, digestion, and utilization of food and systems of feeding; growth, reproduction, and all physiological processes concerned with the use of food; the relation of diet to health and disease; the feeding of livestock.

Each number contains also a Review Article dealing with some subject of current practical importance.

Published quarterly. Price 105*s*. per volume. The current volume is Vol. 27.

TECHNICAL REPORTS

From time to time the Bureau prepares Technical Communications on subjects of practical importance. A list of these may be had on request.

CONSULTATION SERVICE

The Bureau is prepared, within the limits of its information, to answer enquiries from workers in laboratory research, field investigations, or nutritional planning, who do not themselves have the necessary access to literature.

Notes to Contributors

Manuscripts

(i) ***General***

Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts.* All citations should be checked with the original articles, and each one referred to in the text by the key number.

(iv) ***Tables***

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should be used only when they are essential. Numerous small tables should be avoided.

Illustrations

(i) ***General***

All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

(ii) ***Line drawings***

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used unless it is desired to have all the co-ordinate lines show. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. In large drawings or groups of drawings the ratio of height to width should conform to that of a journal page but the height should be adjusted to make allowance for the caption.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard with no space or only a **very** small space (less than 1 mm.) between them. In mounting, full use of the space available should be made (to reduce the number of cuts required) and the ratio of height to width should correspond to that of a journal page (4¾ × 7½ in.); however, allowance must be made for the captions. Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. An additional charge is made for illustrations that appear as coated inserts. The cost per page is given on the reprint requisition which accompanies the galley.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

Contents

Page

The Antiaccelerator Effect of Veratramine on the Isolated Perfused Heart of the Cat—*Maurice F. Murnaghan* - - - - - - - - - - 173

The Effect of Ingested Chlortetracycline on Some Hydrolases and Organs Associated with the Digestive Process in Growing Pigs. I. Assay Methods for Protease, Amylase, and Cellulase Activity—*M. A. A. Vonk, L. W. McElroy, and R. T. Berg* - - - - - - - - - - - - - 181

The Effect of Ingested Chlortetracycline on Some Hydrolases and Organs Associated with the Digestive Process in Growing Pigs. II. Pancreas—Dry Weight, Fat Content, Protease, and Amylase Activity—*M. A. A. Vonk, L. W. McElroy, J. P. Bowland, and R. T. Berg* - - - - - - 187

The Effect of Ingested Chlortetracycline on Some Hydrolases and Organs Associated with the Digestive Process in Growing Pigs. III. Protease, Amylase, and Cellulase Activity of the Contents of the Small Intestine and of the Cecum; Weights of Small Intestine and Mucosa—*M. A. A. Vonk, L. W. McElroy, and R. T. Berg* - - - - - - - - - - 195

Seasonal Fluctuations in Blood Volume—*J. Doupe, M. H. Ferguson, and J. A. Hildes* - - - - - - - - - - - - - - - - 203

The Effect of BZ-55 (Carbutamide) on the Glucose-6-phosphatase Activity in the Livers of Intact and Alloxanized Female Rats—*R. D. Hawkins and R. E. Haist* - - - - - - - - - - - - - - - 215

Biosynthesis of Quercetin in Buckwheat. Part I.—*E. W. Underhill, J. E. Watkin, and A. C. Neish* - - - - - - - - - - - - - 219

Biosynthesis of Quercetin in Buckwheat. Part II.—*J. E. Watkin, E. W. Underhill, and A. C. Neish* - - - - - - - - - - - - - 229

Notes:

A Note on the Infrared Spectra of α-Cephalins—*Erich Baer* - - - 239